Docket No.: HO-P02703US2

(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Atul Varadhachary et al.

Application No.: 10/728,521 Confirmation No.: 8270

Filed: December 5, 2003 Art Unit: 1656

For: ORAL LACTOFERRIN IN THE TREATMENT OF SEPSIS Examiner: C. M. Kam

ORIGINAL APPEAL BRIEF

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

As permitted under § 1.136(a)(1), this brief is filed within SIX months after the Notice of Appeal, filed in this case on October 2, 2007, and is in furtherance of said Notice of Appeal.

This brief contains items under the following headings as required by 37 C.F.R. § 41.37 and M.P.E.P. § 1206:

I. REAL PARTY IN INTEREST

The real party in interest for this appeal is the assignee, AGENNIX, INC.

II. RELATED APPEALS, INTERFERENCES, AND JUDICIAL PROCEEDINGS

There are no other appeals, interferences, or judicial proceedings which will directly affect, be directly affected by, or have a bearing on the Board's decision in this appeal.

III. STATUS OF CLAIMS

A. Total Number of Claims in Application

Claims 1-44 were originally filed with the Application on December 5, 2003.

B. Current Status of Claims

Prior to examination, claims 1-20 and 26-40 were elected pursuant to a restriction requirement mailed October 20, 2004.

Following an Office Action mailed January 10, 2005, a response was filed on April 11, 2005 canceling claims 2-6, 11-13, 21-25, 33-37, and 41-44; adding claims 45-46; and amending claims 1, 26, 27, 31, 32, and 38. Following an Office Action mailed June 17, 2006, a response was filed on September 19, 2005 amending claims 1, 8-10, 15, 16, 26-28, 31, 32, and 38; and canceling claims 45-46. Following an Office Action mailed December 5, 2005, a response was filed on February 13, 2006 amending claims 1, 8-10, 15, 16, 26, 27, 31, 32, and 38. Following an Office Action mailed April 24, 2006, a response was filed on September 26, 2006 amending claims 1, 26, 27, 31, 32, and 38. Following an Office Action mailed December 6, 2006, a response was filed in which no amendments to the claims were made.

A Final Office Action was mailed on July 5, 2007 rejecting the pending claims 1, 7-10, 14-20, 26-32 and 38-40. Applicant filed a Notice of Appeal on October 2, 2007.

C. Claims On Appeal

Pending claims 1, 7-10, 14-20, 26-32 and 38-40 all stand rejected and all of these claims are subject to this appeal. The claims on appeal are reproduced in Appendix A.

IV. STATUS OF AMENDMENTS

There are no outstanding amendments to the claims.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Page and paragraph numbers refer to the as-filed U.S. Application No. 10/728521. The subject matter of the six independent claims currently appealed (*i.e.* claims 1, 26, 27, 31, 32, and 38) is summarized below. The location of support for terms in earlier claims is the same as, and thus not duplicated in the summary of, later claims.

Independent claim 1 (see claims appendix) claims a method of treating bacteremia, which is defined as having a focus of a bacterial infection or bacteria in the blood (p.8 ¶[0032]) caused by gram-negative or gram-positive bacteria (p.19 ¶[0081]) with primary infection sites of the lungs, GU or GI tract, or soft tissues such as skin, often resulting from surgical manipulation and/or catheterization of infected tissues, or colonization of indwelling devices (p.19 ¶[0082]); the method comprising the step of administering orally to a mammalian subject (p.11 ¶[0052]) an effective amount, *i.e.* an amount that results in an improvement or remediation of the symptoms of the disease or condition (p.8 ¶[0035], p.22 ¶[0091]), of a lactoferrin composition; lactoferrin defined as a single chain, metal binding, glycoprotein (p.3 ¶[0008]) and a lactoferrin composition comprising lactoferrin or an N-terminal lactoferrin variant in which at least the N-terminal glycine residue is truncated or substituted (p.4 ¶[0012]), comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant (*Id.*, p.16 ¶[0066]); to provide an observable or measurable improvement in the bacteremia of the subject, wherein the improvement is selected from the group consisting of attenuating sepsis, attenuating septic shock, attenuating organ failure, decreasing morbidity and decreasing mortality (p.5 ¶[0015], p.12 ¶[0053]); and the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues (p.10 ¶[0046]) and the N-terminal lactoferrin variant also retains the same biological function, *e.g.* the ability to stimulate or inhibit various cytokines or chemokines attenuating sepsis, septic shock, organ failure, decreasing morbidity, and/or mortality, as full length lactoferrin (p.10 ¶[0046], p.14 ¶[0063]-p.15 ¶[0065]).

Independent claim 26 (see claims appendix) claims a method of treating bacteremia or sepsis, sepsis defined as the Systemic Inflammatory Response Syndrome (SIRS) which is a complex immunologic response to an infective process and may include: vasodilation of arteries and arterioles, decreasing peripheral arterial resistance with normal or increased cardiac output, eventual cardiac output and peripheral resistance increase, impaired blood flow to the capillary exchange vessels, and failure of one or more of the visceral organs (p.1 ¶[0003], p.2 ¶[0005]); the response resulting from a bacterial infection that may originate anywhere in the subject's body (*Id.*); the method comprising the step of supplementing the mucosal immune system, *i.e.* increasing the amount of lactoferrin in a subject's gastrointestinal tract (p.5 ¶[0017], p.23 ¶[0095]) by administering orally, *e.g.* orally, buccally, enterally, rectally or intragastricly (p.11 ¶[0047]) an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

Independent claim 27 (see claims appendix) claims a method of enhancing a mucosal immune response, meaning stimulating cytokines and/or chemokines in the subject's gastrointestinal tract which stimulates the activity or production of immune cells while reducing the production or activity of pro-inflammatory cytokines in the subject's gastrointestinal tract (p.5 ¶[0018], p.23 ¶[0096]) comprising the step of administering orally to said subject an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant, wherein the composition results in enhancement of the mucosal immune system wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

Independent claim 31 (see claims appendix) claims a method of decreasing mortality, *i.e.* risk of death (p.22 ¶[0091]) of a subject having bacteremia comprising the step of administering orally to said subject an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant to attenuate the bacteremia to

decrease mortality of said subject (p.6 ¶[0020]), wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

Independent claim 32 claims a method of treating a septic condition in a subject comprising the step of administering orally to said subject an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant to provide an improvement in the septic condition of said subject, wherein the improvement is selected from the group consisting of decreasing the levels of circulating bacteria, attenuating sepsis, attenuating septic shock, attenuating organ failure, decreasing morbidity and decreasing mortality (p.6 ¶[0021]) wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

Independent claim 38 claims a method of decreasing mortality of a subject having sepsis comprising the step of administering orally to said subject an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant to attenuate sepsis to decrease mortality of said subject (p.6 ¶[0022]) wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Were claims 1, 7, 14, 17-20, 26-32 and 38-40 improperly rejected under 35 U.S.C. § 103(a) over Van Bree *et al.* (WO 01/72322) ?

B. Should the provisional rejection of claims 1, 7-10, 14-20, 26-32 and 38-40 under the judicially created doctrine of obviousness type double patenting be held in abeyance?

VII. ARGUMENT

A. The Claims Are Patentable Over Van Bree *et al.* (WO 01/72322)

Claims 1, 7, 14, 17-20, 26-32 and 38-40 are patentable over Van Bree *et al.* (WO 01/72322; "Van Bree;" **EXHIBIT A**), thus the rejection under 35 U.S.C. § 103(a) must be reversed. As fully explained below, after a determination of the scope and contents of the prior art, a comparison of the differences between the prior art and the claims at issue, and a resolution of the level of ordinary skill in the art, the Examiner has failed to show that Appellant's claims are *prima facie* obvious in view of the prior art because (1) ***the prior art does not teach all of Applicant's claim limitations***, and (2) ***no rationale to support an obviousness rejection is presented***. M.P.E.P. § 2143.03; *In re Royka*, 490 F.2d 981, 180 U.S.P.Q. 580 (CCPA 1974); Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in *KSR International Co v. Teleflex Inc.*, 72 Fed. Reg. 57,526 (Oct 10, 2007); *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966); *KSR Int'l Co. v. Teleflex Inc.*, 550 U. S. ____, 127 S. Ct. 1727, 1741 (2007). The Supreme Court has held that demonstrating the several claimed elements exist in the prior art is insufficient to find obviousness, rather, reasons for prompting a person of skill in the art to combine those claimed elements in the claimed fashion must be identified. *KSR*, 127 S. Ct. at 1741. Here, the Examiner has failed to demonstrate either the existence of the elements in the prior art, or a reason for combining them, thus the burden of showing *prima facie* obviousness is not met. M.P.E.P. § 2142 ("The initial burden is on the examiner to provide some suggestion of the desirability of doing what the inventor has done.").

1. *Graham* Factors: The Scope and Contents of the Prior Art

Van Bree is the sole prior art reference cited in the Final Office Action dated July 5, 2007 ("Final Action"), as well as four of the five preceding non-final Actions. The Examiner concedes and Appellant agrees that Van Bree does not provide any examples of specific methods of treating bacteremia, enhancing a mucosal immune response, or decreasing mortality using a lactoferrin composition containing an N-terminal variant. Final Action, p.3. However, the Examiner also asserts that (1) Van Bree teaches oral administration of lactoferrin ("LF") and variants thereof; (2) Van Bree teaches N-terminal LF variants having the same biological function as natural LF; and (3) LF and N-terminal variants thereof can treat large scale (bacterial) infections, blood borne infection (sepsis), and inflammation due to infection provided the composition administered is 1-20% LF or LF variant. Final Action, p.2-3. As explained below, this characterization of Van Bree is simply incorrect.

Van Bree Does Not Teach Oral Administration Without Parenteral

When read as a whole in view of knowledge in the art, it is clear that Van Bree's teaching regarding oral administration of LF and LF variants is extremely narrow, does not suggest broader possibilities, and is limited to supplementing high dosage parenteral administration. Beginning with the Introduction, Van Bree describes, "methods of treating human patients by ***parenteral*** administration of relatively high dosages of lactoferrin," which have few side effects in part because of the, "ease of purification of pharmaceutical grade lactoferrin for ***parenteral*** administration," and "LF and fragments or variants of LF are used at high doses, with a lack of adverse side effects, to treat diseases and conditions that require a ***bolus of and/or sustained large doses***." Van Bree, p.3 ll. 3-16 (emphasis added). It is known in the art that parenteral administration excludes administration via the digestive tract, but includes intravenous or intramuscular injection, and a bolus is a large dose of a drug typically given intravenously for rapid therapeutic delivery into the bloodstream.

Van Bree also describes indications for the administration of LF and/or LF variants, but ***only parenteral administration*** is mentioned. Van Bree, p.18 ll. 31-33 ("High dosage ***parenteral*** administration of lactoferrin or a pharmaceutical composition containing the same

can be used in a variety of therapeutic and prophylactic applications.") (emphasis added). With particular regard to sepsis, Van Bree clearly indicates that sepsis is a condition that ***requires*** a bolus of LF and/or sustained large doses of parenterally administered (i.e. systemic) LF for therapeutic effect. Van Bree, p.3 ll. 3-7 and 13-18; p.18 ll. 31-33 and p.20 ll. 24-25.

After again emphasizing parenteral administration, Van Bree discloses oral administration as optionally used in conjunction with parental administration. Specifically, Van Bree states that, "such pharmaceutical compositions are usually administered ***parenterally***, ***preferably intravenously. . . . Oral administration can also be used***, optionally but not necessarily, ***in conjunction with parenteral administration***." Van Bree, p.23 ll. 16-22. The limited use of oral administration is also reflected by Van Bree's claims, which only include oral administration as a second dosage given with a first, independently claimed (thus implicitly effective), parenteral dose . Van Bree, claims 13-15 as filed (below):

> 13. A method of treating a patient, comprising parenterally administering a dosage of lactoferrin or a fragment variant thereof, of at least 139 nmol/kh body weight to the patient.
>
> 14. The method of claim 13, wherein the administering is performed by intravenous injection.
>
> 15. A method according to claims 13 or 14, further comprising administering a second dosage of lactoferrin or a fragment variant thereof, orally to the patient.

Clearly, the above shows that Van Bree teaches oral administration only for the limited purpose of supplementing parenteral administration.

Furthermore, it was known in the art that LF is not systemically bioavailable when ingested; even high oral doses of LF do not significantly raise plasma LF levels. Specification, p.2-3 ¶[0007]; Kuhara *et al.*, "Orally Administered Lactoferrin Exerts an Antimetastatic Effect and Enhances Production of IL-18 in the Intestinal Epithelium," *Nutrition and Cancer*, Vol 28 No 2, 2000, pp. 192-199 at pg. 197, lines 8-10; **EXHIBIT B**. Thus, one of skill in the art would not read Van Bree and believe that oral administration could provide the same benefits of systemic LF provided parenterally in high dosages. Rather, one of skill in the art would

understand that Van Bree teaches parenteral administration, which may be supplemented with, but not replaced by, oral administration.

N-terminal Lactoferrin Variants Having Full-length Activity Is Not Shown

Van Bree teaches LF variants for the treatment of certain diseases and conditions, and such variants are described. Van Bree, p.3 ll. 28-30; p.4-7. The Examiner argues that pages 4, 5, and 27 of Van Bree disclose N-terminal LF variants having the same biological activity (*e.g.* effective in killing virus and bacteria) of natural LF where the N-terminal amino acids 2-5 are deleted or mutated to remove the positive charge(s). However, Van Bree does not teach N-terminal LF variants with the same biological function as Appellants describe for full length LF.

Initially, Appellants note that Van Bree lists effective killing of viruses or bacteria as examples of antimicrobial activity, not examples of LF functional equivalence. Van Bree, p.27 ll. 12-14. To compare, certain biological and/or functional activity of an LF protein ***that is retained*** in an N-terminal LF variant is described in Appellant's Specification, and includes: stimulating the production of various cytokines (e.g., IL-18, MIP-3α, GM-CSF or IFN-γ), inhibiting various cytokines (e.g., IL-2, IL-4, IL-5, IL-6, IL-10, and TNF-α.), attenuating sepsis, attenuating septic shock, attenuating organ failure, decreasing morbidity, and/or decreasing mortality. ¶¶[0046], [0060]-[0064]. Thus, the Examiner has not shown that Van Bree teaches variants with the full length LF activity Appellants describe.

Van Bree Does Not Teach Oral Lactoferrin Administration to Treat Sepsis.

Finally, Van Bree does not teach treatment of bacteremia and/or sepsis with an effective amount of orally administered LF. Regarding indications, Van Bree discloses LF administration for therapy or prophylaxis of infection, including local, large scale, and blood borne infection, as well as inflammation resulting from infectious or non-infectious inflammatory diseases. Van Bree, p.20 ll. 24-27. Van Bree also teaches that the "particular form of the composition varies with the intended mode of administration and therapeutic application." *Id.*, p.27 ll. 9-10. As explained above, Van Bree discloses oral administration as optionally supplementing parenteral administration. Yet, there is no suggestion or teaching in Van Bree to use an effective amount of

oral LF in the treatment of bacteremia or sepsis. The sole disclosure related to therapeutic oral LF administration is directed to "infections or disorders of the digestive tract" of which bacteremia and sepsis are not examples. Van Bree, p.26 ll. 22-24. Treatment of bacterial infection is through intravenous administration of LF at high dosages. Van Bree, p.3 ll. 14-31; p.22 ll. 7-12. Appellants also note that Van Bree's working examples only observe the effect and safety of rhLF on the neutralization of heparin via intravenous rhLF, and thus do not teach treatment of infection at all. Van Bree, pp.29-35. In sum, ***oral administration*** of an ***effective amount of LF*** for treatment of ***bacteremia or sepsis*** is ***not taught*** by Van Bree.

2. *Graham* Factors: Ascertaining the differences between the prior art and the claims in issue

As indicated at least by the above, the sole prior art reference differs from the claimed invention in several significant ways. First, ***the claimed invention requires oral administration of an effective amount*** of LF. In contrast, Van Bree teaches oral administration for the limited purpose of supplementing the preferential method of administration: parenteral, which is known to not be interchangeable with oral administration, because LF is not systemically bioavailable when ingested. This is a key difference because ***Van Bree teaches that to have a therapeutic effect on sepsis, high dose systemic LF is required***, thus teaching away from oral LF administration in a therapeutically effective amount. Van Bree, p.3 ll. 3-7 and 13-18; p.18 ll. 31-33 and p.20 ll.24-25. Secondly, Van Bree teaches ***N-terminal variants having different biological function*** as full length LF, while ***the claimed invention requires the same biological activity***. To compare, Appellant's claims are for methods of treating bacteremia, sepsis, septic conditions, decreasing mortality, and/or enhancing a mucosal immune response comprising at least the step of administering orally to a subject an effective amount of a LF composition.

"In determining the differences between the prior art and the claims, the question under 35 U.S.C. 103 is not whether the differences ***themselves*** would have been obvious, but whether the claimed invention ***as a whole*** would have been obvious." M.P.E.P. § 2141.02, citing *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 218 USPQ 871 (Fed. Cir. 1983). This is particularly pertinent when, as here, the inventor's insight is contrary to the understandings and expectations of the art. MPEP § 2141; *Schenck v. Nortron Corp.*, 713 F.2d 782, 218 USPQ 698

(Fed. Cir. 1983) (holding that the invention made integral what had been four separate but bolted pieces, yet, "that insight was contrary to the understandings and expectations of the art, [thus] the structure effectuating it would not have been obvious to those skilled in the art."). One of skill would have no impetus to modify the prior art into something closer to the claimed invention as a whole because the knowledge in the art taught such changes (*e.g.* orally administered LF) would not work. Thus, the prior art does not include all elements of the claimed invention as a whole.

3. No Rationale To Support Obviousness is Provided, thus the Examiner's Burden is Not Met

As shown above, the Examiner has failed to show that Appellant's claims are *prima facie* obvious in view of the prior art because the prior art does not teach ***all*** of Applicant's claim limitations, including "administering orally to a subject an effective amount of a lactoferrin composition" and methods of using N-terminal LF variants "wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin." M.P.E.P. § 2143.03; *In re Royka*, 490 F.2d 981, 180 U.S.P.Q. 580 (CCPA 1974). Further, the United States Supreme Court has recently made clear that a reasoned basis justifying the legal conclusion of obviousness must be made by the Examiner. "Rejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *KSR Int'l Co. v. Teleflex Inc.*, 550 U. S. ____ (2007), 127 S. Ct. 1727, 1741 (2007). However, the Examiner has failed to provide such reasoning here.

At most, the Examiner has indicated that oral administration of LF ***may*** be advantageous because ***if*** produced by a transgenic animal (*e.g* a cow), that excretes the LF in a human-consumable form (*e.g.* milk), it ***may*** be administered sans extensive purification. Final Action, p.3; Van Bree, p.26 ll. 16-21. Yet, the Supreme Court has held that this is not sufficient. The Examiner may have intended to argue that oral administration of a pharmaceutical was a known technique or element, applied to a known or similar method or element, but each of these basis for an obviousness rejection also requires a reasonable expectation of success or outright predictable results. 72 Fed. Reg. at 57,529. Considering that it was known that ingested LF is

not systemically available, and in view of Van Bree's teaching regarding the requirement that LF be made systemically available in high dosages, there can be neither a reasonable expectation of success nor the necessary measure of predictability in the Examiner's advanced modification. Thus, the Examiner's burden is not met and the rejection under 103 must be reversed.

B. The Provisional Double Patenting Rejection Should be Held in Abeyance until Made Non-provisional.

The Examiner provisionally rejected claims 1, 7, 14, 17-19, 26-32 and 38-40 under the judicially created doctrine of obviousness-type double patenting over claims 16-22, 26-30 and 50-51 of co-pending U.S. Application No. 10/663,258. A "provisional rejection" is contingent upon an event unrelated to the prosecution of the present application that may or may not come to pass. Because the co-pending application may never actually issue, or, alternatively, the claims as originally filed may be amended during the course of prosecution of the co-pending application such that the claims, when issued, no longer impact Applicants' claims, the rejection remains "provisional" until the co-pending application issues. If the issued application still results in a double-patenting rejection, the provisional status of the rejection is removed, and the rejection becomes a non-provisional double-patenting rejection.

Once the rejection is made non-provisional, Applicants are then able to address fully the double-patenting concerns with regard to the substance of the claims that have actually issued in the referenced patent. The Office and Applicants are saved from needless speculation as to whether the co-pending application will actually issue or what the substance of the final issued claims will be. As such, Applicants are not required to address the merits of the provisional double-patenting rejections until such time as the co-pending applications actually issue.

In the Final Action, the Examiner noted Appellant's acknowledgement of the provisional double-patenting rejection, but sustained its provisional nature in view of the remaining rejection which Appellant addressed fully above. Final Action, p.7. Further, Appellant notes that the cited co-pending application has not issued, and currently remains in prosecution. *See*, **EXHIBIT C**. Thus, it is proper for The Board to address the merits of Appellant's arguments above and withhold opinion as to the double patenting rejection without finding Appellant concedes to the propriety of the rejection as ***Appellant explicitly herein does not concede***.

Prosecution, including appeal, should continue while a provisional double-patenting rejection is pending and the Office may continue to make such a provisional rejection until one of the applications issues as a patent. M.P.E.P. § 804. The courts have also endorsed the appropriateness of the Office allowing the provisional rejection to remain standing during

prosecution until the co-pending application actually issues. For example, in *In re Wetterau*, 148 U.S.P.Q. 499 (C.C.P.A. 1966), the Court of Customs and Patent Appeals held that a provisional double-patenting rejection in view of a co-pending application was proper. Even though the claims of the co-pending application had already been allowed, the court stated, "no assurance can be given that the status will endure and a patent containing such claims will ultimately issue." 148 U.S.P.Q. at 501. The court found the uncertainty of the co-pending application's status was sufficient to allow the provisional rejection to remain standing and to prevent the applicant from having to abandon the case. In holding such, the court stated:

> If a patent were not to issue on the Carabateas [co-pending] application, the "double patenting" rejection, if here affirmed, would of necessity evaporate for the possibility of two patents would not exist. Grave injury to applicant's rights might occur if the Wetterau [applicant's] application were to go abandoned through no fault of the applicant prior to the issuance of Carabateas, the reference application. *Id.*

In view of the above, Appellant submits that it has acknowledged the provisional double-patenting rejection, the Examiner has noted this acknowledgement, and that Appellant is not required to address the merits of the provisional double-patenting rejections until such time as the co-pending application issues and the rejection made non-provisional. Appellant has further shown that no inference should be drawn regarding concession to the rejection from Appellant's response.

CONCLUSION

Appellant has shown above that the pending rejection under 35 U.S.C. § 103 is without merit and should be reversed. Appellant as further shown that the pending provisional double patenting rejection need not be decided until made non-provisional. It is therefore respectfully requested that the Board overturn the 35 U.S.C. § 103 rejection and recommend that this application proceed to allowance upon acceptable resolution of the provisional rejection.

Dated: April 2, 2008

Respectfully submitted,

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VIII. CLAIMS APPENDIX

Appealed Claims:

1. A method of treating bacteremia comprising the step of administering orally to a subject an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant to provide an improvement in the bacteremia of said subject, wherein the improvement is selected from the group consisting of attenuating sepsis, attenuating septic shock, attenuating organ failure, decreasing morbidity and decreasing mortality, wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

7. The method of claim 1, wherein said lactoferrin composition is dispersed in a pharmaceutically acceptable carrier.

8. The method of claim 1, wherein said lactoferrin composition further comprises mammalian lactoferrin.

9. The method of claim 8, wherein said lactoferrin composition further comprises human or bovine lactoferrin.

10. The method of claim 1, wherein said lactoferrin composition further comprises recombinant lactoferrin.

14. The method of claim 1 further comprising administering an antacid in conjunction with said lactoferrin composition.

15. The method of claim 1, wherein the lactoferrin composition further comprises lactoferrin and wherein the amount of lactoferrin plus N-terminal lactoferrin variant in the lactoferrin composition that is administered is about 1 mg to about 100 g per day.

16. The method of claim 1, wherein the lactoferrin composition further comprises lactoferrin and wherein the amount of lactoferrin plus N-terminal lactoferrin variant in the lactoferrin composition that is administered is about 10 mg to about 10 g per day.

17. The method of claim 1, wherein said composition that is administered is a liquid formulation.

18. The method of claims 1, wherein said composition that is administered is a solid formulation.

19. The method of claim 1, wherein said composition that is administered is a solid formulation with an enteric coating.

20. The method of claim 1, wherein oral administration is via a nasogastric tube.

26. A method of treating bacteremia or sepsis comprising the step of supplementing the mucosal immune system in a subject by administering via an oral route an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

27. A method of enhancing a mucosal immune response in the gastrointestinal tract in a subject comprising the step of administering orally to said subject an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant, wherein the composition results in enhancement of the mucosal immune system wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

28. The method of claim 27, wherein said lactoferrin composition stimulates interleukin-18 in the gastrointestinal tract.

29. The method of claim 28, wherein interleukin-18 stimulates the production or activity of immune cells.

30. The method of claim 28, wherein said lactoferrin composition reduces the production or activity of pro-inflammatory cytokines.

31. A method of decreasing mortality of a subject having bacteremia comprising the step of administering orally to said subject an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant to attenuate the bacteremia to decrease mortality of said subject wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

32. A method of treating a septic condition in a subject comprising the step of administering orally to said subject an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant to provide an improvement in the septic condition of said subject, wherein the improvement is selected from the group consisting of decreasing the levels of circulating bacteria, attenuating sepsis, attenuating septic shock, attenuating organ failure, decreasing morbidity and decreasing mortality wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

38. A method of decreasing mortality of a subject having sepsis comprising the step of administering orally to said subject an effective amount of a lactoferrin composition comprising at least 1% to at least 50% w/w of an N-terminal lactoferrin variant to attenuate sepsis to decrease mortality of said subject wherein the N-terminal lactoferrin variant has a deletion, substitution, or combination thereof, of from 1 to 16 N-terminal amino acid residues and wherein the N-terminal lactoferrin variant retains the same biological function as full length lactoferrin.

39. The method of claim 38, wherein the amount of the lactoferrin composition reduces the levels of circulating cytokines.

40. The method of claim 39, wherein the cytokines are selected from the group consisting of IL-4, IL-6 and IL-10.

IX. EVIDENCE APPENDIX

EXHIBIT A. Van Bree *et al.* (WO 01/72322, October 4, 2001); made of record by way of information disclosure statement file February 25, 2004.

EXHIBIT B. Kuhara *et al.*, "Orally Administered Lactoferrin Exerts an Antimetastatic Effect and Enhances Production of IL-18 in the Intestinal Epithelium," Nutrition and Cancer, Vol. 28 No 2, 2000, pp. 192-199; made of record by way of information disclosure statement file April 30, 2007.

EXHIBIT C. U.S. Application No. 10/663,258 PAIR status page, retrieved from U.S.P.T.O website at www.uspto.gov; April 2, 2007.

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(54) Title: HIGH DOSAGE PARENTERAL ADMINISTRATION OF LACTOFERRIN

(57) Abstract: The invention provides methods of treatment using high dosages of lactoferrin. Lactoferrin can be administered parenterally at high dosages without significant side effects to treat a varient of disorders including infectious diseases and inflammation.

High Dosage Parenteral Administration of Lactoferrin

FIELD OF THE INVENTION

The present invention relates to the production, purification and use of recombinant human lactoferrin for parenteral administration in various therapeutic applications.

BACKGROUND

Lactoferrin (LF) is a metal binding glycoprotein of Mr 77,000 found in milk, tears, saliva, bronchial, intestinal, vaginal and other secretions. LF is also present in the secondary granules of neutrophils. Lactoferrin plays an important role in numerous inflammatory and immune response functions such as regulation of monocyte colony stimulating factor synthesis, regulation of interleukin synthesis, activation of natural killer cell activity, inhibition of metastasis, and maturation of T-cells. Lactoferrin also inhibits myelopoiesis, binds to members of the low density lipoprotein receptor family, and blocks the clearance of lipoprotein chylomicron remnant particles (Sanchez, L. *et al.* (1992) *Arch Dis. Child.* 67:657, Iyer, S.*et al.* (1993) *Eur. J. Clin. Nutr.* 47:232, Huettinger, M.*et al.* (1992) *J. Biol. Chem.* 267:18551, Willnow, T.E. *et al.* (1992) *J. Biol. Chem.* 267:26172). It also appears to play a role in inhibiting the production or release of prostaglandin E_2, interleukins, and tumor necrosis factor by mononuclear cells (Bartal, L. *et al.*. (1987) *Pediatr. Res.* 21:54-57, Zucali, J.R.*et al.* (1989) *Blood* 74:1531,Crouch, S.P.M.*et al.* (1992) *Blood* 80:235).

Human LF (hLF) is also a major component of the non-specific defense of mucosal surfaces and neutrophils and is active against a variety of pathogens (reviewed in Nuijens, J.H.*et al.* (1996) *J. Mammary Gland Biol. Neoplasia.* 1:285 and Sanchez, L. *et al.* (1992) *Arch Dis. Child.* 67:657). This protein displays antimicrobial properties against Gram-positive and Gram-negative bacteria by limiting the availability of environmental iron (Bullen, J.J. (1981) *Rev. Infect. Dis.* 3*:1127*). However, since iron-saturated hLF is also able to kill certain bacteria (Ellison, R.T. (1994). *Adv. Exp. Med. Biol.* 357:71), mechanisms other than iron-depletion apparently are involved in the antibacterial activity of lactoferrin.

Some of the biological activities of LF may instead arise from its capacity to bind to other molecules. Direct intermolecular interactions between hLF and human lysozyme (hLZ) may explain the synergy between the antibacterial action of these two

proteins. Interaction of hLF with bacterial outer membrane components such as lipopolysaccharide (LPS) and porins presumably plays an important role in the antimicrobial activity of hLF. Binding of hLF to the lipid A portion of LPS inhibits the LPS priming of neutrophils for enhanced fMLP-triggered superoxide release. Interaction of LF with heparin may account for the neutralization of the anticoagulant activity of heparin.

Some biological activities of LF arise from interactions between LF and cells via membrane bound receptors. For example, LF binding to specific receptors on monocytes, macrophages and activated lymphocytes results in inhibition of cytokine production. Cells that exhibit specific binding to hLF include liver cells, intestinal cells, mammary gland epithelial cells, monocytic cell lines, activated lymphocytes, and platelets.

The amino acid sequence of LF has been determined by protein sequencing and sequencing of cDNA clones. hLF consists of a polypeptide chain of 692 amino acids. The amino terminal region of hLF contains two clusters of basic residues, RRRR (residues 2-5) and RNMRKVR (residues 25-31), whereas bovine LF (bLF) has only one cationic domain (residues 17-42 (Tomita, M. *et al.* (1991) *J. Dairy Sci.* 74:4137, Hoek, K.S. *et al.* (1997) *Antimicrob. Agents Chemother.* 41:54)). The LF polypeptide is folded into two globular lobes, each of which contains an iron-binding cleft. The high affinity of LF for iron confers to the protein certain antibacterial properties and, in addition, may play a role in the absorption of dietary iron by the small intestine.

There have been a number of studies to assess the therapeutic potential of human lactoferrin (see e.g., Boxer et al., (1982) J. Lab. Clin. Methods 99, 866-872; Kurose et al., (1994) J. Leukoc. Biol. 55, 771-777; Levergule et al., Eur. J. Biochem. 213, 1205-1211 (1993)) These studies have indicated that at least in some circumstances, parenteral administration of lactoferrin results in significant side effects including neutropenia, intestinal mucosal injury and vascular leakage and tissue damage.

DETAILED DESCRIPTION

I. Introduction

The invention provides methods of treating human patients by parenteral administration of relatively high dosages of lactoferrin. The invention is premised, in part, on results that demonstrate that dosages of at least 60 mg/kg body weight of human lactoferrin can be safety administered to human patients (see Examples). These results are in contrast to the significant side effects reported in references discussed in the Background section. Although practice of the invention is not dependent on an understanding of mechanism, it is believed that the lack of side effects in the present methods results, in part, from the large amounts of human lactoferrin available to the present inventors through the production of lactoferrin in milk of transgenic cattle, and the consequence ease of purification of pharmaceutical grade lactoferrin for parenteral administration. The ability safely to administer high dosages is advantageous in a number of therapeutic applications. Intact LF and fragments or variants of LF are used at high doses, with a lack of adverse effects, to treat diseases and conditions that require a bolus of and/or sustained large doses. Some diseases and conditions that can be treated are gastroenteritis, Inflammatory Bowel Diseases, sepsis, ARDS, MOF, GVD, GVHD and systemic inflammation. Large doses, in the absence of adverse effects, allow the blocking or neutralizing of inflammatory agents and/or boost the clearance of inflammatory agents. IBD (ulcerative colitis and Crohn), for instance is known to cause leakage of the gut allowing LPS or other infectious agents to pass through into the blood system. Administering hLF can block these free LPS or other infectious or inflammatory agents and cause them to clear from the body more rapidly and/or to mask their inflammatory activity, thus rendering them harmless. By forming a complex with hLF, inflammatory agents are cleared more quickly through the liver. Additionally, by increasing membrane permeability, hLF assists the passage of antibiotics, thus potentiating their antibacterial effects (see Kuipers et al., Antimicrobial Agents and Chemotherapy 43, 2635-2641).

hLF is useful in a variety of therapeutic and prophylactic applications, including use as antimicrobial agents and the treatment of, *e.g.*, inflammation, anemia, myelopoieses and for reducing reperfusion injury, cytokine release, and proteoglycan-mediated entry of virus into cells. LF variants are also useful for treatment of these diseases and conditions, and are especially useful for treatment of those conditions for which beneficial effects of natural LF treatment are due to binding to a high affinity LF receptor. Such LF variants have the biological activities of natural LF, *e.g.*, binding to high affinity LF receptors on

cells, but with reduced binding, relative to natural LF, to heparin, DNA, human lysozyme, the Lipid A component of bacterial lipopolysaccharide (LPS), and sulfated cell surface molecules. Thus, an advantage to the use of the LF variants is that the desired physiological effect can be achieved while avoiding side effects caused by the binding of natural LF to heparin, DNA, human lysozyme, Lipid A, or cell surface proteoglycans. For example, the LF variants can be used to deliver nutritional iron to cells, without concurrent neutralization of heparin and similar effects. Because some LF variants have little or no binding to sulfated cell surface molecules, and bind with increased affinity to high affinity LF receptors, more efficient targeting of LF to these receptors can be achieved.

Such variants include short polypeptides having one or more arginines in the N-terminal segment of the polypeptide, such as found in the first cationic domain of hLF, which have been found to exhibit significant therapeutic activity. Other variants of LF have 1-4 arginine residues from the first basic cluster (*i.e.*, residues 2-5) deleted. Some variants include one or more residues from the first cationic domain of hLF, but not amino acids from the second cationic domain. Some polypeptides are quite short, such as less than 27 amino acids in length. Given their short length, such polypeptides are easily and inexpensively prepared and are readily amenable to use in pharmaceutical compositions.

II. Definitions

The terms "polypeptide," "peptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. Unless otherwise stated, the term also applies to amino acid polymers in which one or more amino acids are chemical analogues of a corresponding naturally occurring amino acid.

The term lactoferrin protein refers generically to intact lactoferrin proteins found in nature, allelic, species and induced variants thereof, and fragments. Induced variants typically show at least 85% amino acid sequence identity over the entire length of the variant to a natural lactoferrin sequence when maximally aligned using the BLASTN algorithm with a wordlength (W) of 3, M=5, and N= -4 (see Altschul *et al., J. Mol. Biol.* 215:403-410 (1990)). Variants preferably differ predominantly or exclusively by conservative mutations from a natural form of human lactoferrin. Fragments typically comprise at least 6, 10, 20, 50, 100 or 200 contiguous amino acids from a natural lactoferrin sequence. Examples of natural human lactoferrin sequences are provided in

SEQ ID NO:1, by Powell, M.J. and Ogden, J.E., Nucleic Acids Res. 18:4013 (1990), which is incorporated herein by reference, Rado, T.A., *et al.*, Blood 70:989-993 (1987), and Heyneker, H.L., WO 91/08216, each of which is incorporated herein by reference. Some lactoferrin proteins have a modified protein backbone. Examples of such modifications include acetylations, carboxylation, glycosylation modifications and other processing variants of hLF. Some lactoferrin variants comprise polypeptides having the sequence of natural LF from which 1, 2, 3 or 4 arginine residues at the amino terminus have been removed (*i.e.*, deletion of all or part of the first basic cluster) or from which the residues of the second basic cluster have been removed, or from which both the first and second basic clusters have been removed. Other LF variants have a deletion of the second basic cluster and deletions of one or more amino-terminal arginine residues. Still other LF variants are hLF from which the residues of the second basic cluster have been mutated (*e.g.*, to uncharged residues). The amino-terminal sequence of hLF is: N'-GRRRSVQWC. Some LF variants include a variant having a deletion of one arginine (along with the terminal glycine) residue (referred to as hLF-2N), a variant having two arginine residues removed (referred to as hLF-3N), a variant having three arginine residues removed (referred to as hLF-4N), and a variant having all four arginine residues removed (referred to as hLF-5N). The arginine residues of the first basic cluster can be removed by proteolysis of natural LF or by expression of a polynucleotide encoding a truncated hLF. Alternatively, one or more arginine residues of the first basic cluster can substituted for by other (*i.e.*, other than arginine) amino acids, *e.g.* , by directed mutagenesis of a polynucleotide encoding hLF. In some variants, one or more arginine residues of the first basic cluster are deleted or mutated to an amino acid that is not positively charged at physiological pH, *i.e.*, a neutral or acidic residue, usually to a neutral amino acid, most often alanine, leucine, glycine, valine or isoleucine. Hereinafter, reference to a hLF variant from which all or some of the arginine residues the first basic cluster have been "deleted" or "removed" refers both to removal of the arginines of the first basic cluster by deletion or by mutagenesis, unless stated otherwise.

The term "naturally-occurring" as applied to an object refers to the fact that an object can be found in nature. Natural lactoferrin includes recombinantly encoded hLF ("rhLF") expressed in a transgenic non-human animal, such as a bovine, where the glycosylation pattern may be distinct from glycosylation patterns of naturally occurring hLF obtained from human milk. Natural hLF includes recombinantly encoded hLF expressed in a transgenic non-human animal, such as a bovine, where the glycosylation

pattern may be distinct from glycosylation patterns of naturally occurring hLF obtained from human milk.

"Neutralized lactoferrin" is LF having substantially the sequence of native LF but that, by virtue of modification of the residues of the first basic cluster, is not able to bind to a LF ligand, *e.g.*, heparin, as measured by solid phase ligand binding assay, but still binds 105 kD LF receptor found on Jurkat human lymphoblastic T-cells (Bi *et al.* (1994) *Eur. J. Cell Biol.* 65:164 and Bi *et al.* (1996) *Eur. J. Cell Biol.* 69:288). "Modification" includes chemical modification of the residues of the first basic cluster or, alternatively, binding of a molecule that blocks (*i.e.*, through steric hinderance) the interaction of the first basic cluster of LF and heparin. Blocking molecules include monoclonal antibodies, fragments thereof, and LF ligands such as human lysozyme or heparin.

In a shorthand format for referring to subsequences of hLF, the specific residues being referred to are placed in parentheses. For example, hLF(1-11) refers to residues 1 to 11 inclusively from the N-terminus of hLF; similarly, hLF(2-11) refers to residues 2 to 11 inclusively from the N-terminal region of hLF. hLF (*i.e.*, the full-length protein) that lacks a certain number of residues from the N-terminus is referred to as hLF^{-xN}, where x is the number of N-terminal residues missing. Thus, for example, hLF in which the N-terminal glycine and arginine are removed is referred to as hLF^{-2N}; hLF missing the N-terminal glycine and the two N-terminal arginines, is referred to as hLF^{-3N}, and hLF lacking the N-terminal glycine and first three arginine residues is referred to as hLF^{-4N}. The three arginines located at the N-terminus (*i.e.*, residues 2, 3 and 4 of SEQ ID NO:1) are referred to as Arg^2, Arg^3 and Arg^4, respectively. Unless otherwise stated, the N-terminal amino acid of hLF refers to Gly^1 (see SEQ ID NO:1); the 31 residues located at the amino-terminus of hLF are: N'-GRRRRSVQWCAVSQPEATKCFQWQRNMRKVR (residues 1-31 of SEQ ID NO:1).

A "conservative substitution," when describing a protein, refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity. Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (*e.g.*, acidic, basic, positively or negatively charged, polar or non-polar) such that the substitutions of even critical amino acids do not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well-known in the art. *See, e.g.*, Creighton (1984) *Proteins*, W.H. Freeman and Company. In addition, individual

substitutions, deletions or additions, which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations."

The phrases "specifically binds to a protein" or "specifically immunoreactive with," when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, a specified antibody binds preferentially to a particular protein and does not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. A molecule such as an antibody that specifically binds to a protein has an association constant of at least 10^6 M^{-1} or 10^7 M^{-1}, preferably 10^8 M^{-1} to 10^9 M^{-1}, and more preferably, about 10^{10} M^{-1} to 10^{11} M^{-1} or higher. A variety of immunoassay formats can be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. *See*, *e.g.*, Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

Intact human lactoferrin, its fragments and variants are "substantially free" of other human proteins when at least about 90%, more usually at least about 95%, and most commonly at least about 99% of the *human* protein present in the sample is the human lactoferrin, fragment or variant. The amount of any specific protein present in a sample can be determined by quantitative SDS-PAGE (for relatively simple mixtures) or by immunological assays (*e.g.*, ELISA and RIA) for more complex mixtures (*e.g.*, a mixture of bovine milk proteins and LF variant).

"Substantially pure" means an object species is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition comprises more than about 80 to 90 percent, and preferably 95%, 99%, 99.5% or 99.9% of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the

composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

Immunological and molecular biological methods are well known and are described, for example, in Sambrook *et al.*, Molecular Cloning - A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 and Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York (1988), both of which are incorporated herein in their entirety and for all purposes.

The term "patient" includes human and veterinary subjects.

III. Production of lactoferrin and lactoferrin polypeptides and variants

A. Purification and production of LF

LF is abundant in milk and is most easily purified from this source, although it is also found in exocrine secretions and secondary granules of neutrophils. A preferred source of hLF is milk from a transgenic bovine species containing a hLF transgene (see section III, C). The transgene-encoded hLF is substantially purified from other milk proteins in the milk of transgenic cows, and is preferably substantially isolated from endogenous bovine LF, if present in the milk. Numerous methods for purification of hLF from milk have been reported. See, for example, U.S. Patents 4,436, 658; 4,791,193; and 4,668,771, which are incorporated herein by reference. See also, Nuijens *et al. J., 1996, Mammary Gland Biology and Neoplasia* 1:3, 283-293 (1996) and references cited therein.

A preferred method for hLF purification is described PCT Application PCT/EP95/00583, which is incorporated herein by reference. Briefly, milk or a milk fraction containing hLF is contacted with a strong cation exchange resin (*e.g.*, S Sepharose™) in the presence of relatively high ionic strength (0.2M to 0.5M NaCl or KCl, preferably 0.4M NaCl or KCl) to prevent binding of non-LF proteins and other substances to the strong cation exchange resin and to reduce electrostatic interactions of LF with other proteins (e.g., caseins) or substances (e.g., lipopolysaccharide), and to liberate LF from complexes. The strong cation exchange resin containing the bound LF is separated from the unbound compounds in the milk or milk fraction, typically by centrifugation or sedimentation followed by batchwise washing and/or by pouring the resin into a column and washing the beads with buffer having approximately equal or lower salt concentration. The LF bound to the cation exchange resin is eluted with an aqueous, typically buffered, NaCl or KCl gradient (e.g., linear gradient of 0-1M NaCl in 20 mM sodium phosphate, pH 7.5) or by batch elution or stepwise elution with an

aqueous, preferably buffered, NaCl or KCl solution of 0.4M or greater, preferably at least 0.5M NaCl or KCl. By selecting appropriate elution conditions, hLF can be substantially purified from bovine milk and substantially separated from bovine LF by an efficient method.

hLF (e.g., rhLF) can be further purified from endogenous LF (e.g., bLF) by the additional subsequent step of rechromatography on a strong cation exchanger, such as S Sepharose™ Fast Flow, with salt gradient or stepwise elution to separate hLF from remaining traces of endogenous non-human LF species (e.g., bLF), and/or can optionally include affinity chromatography with a concanavalin A resin to further separate hLF from bLF, with bLF being more strongly bound to the Con A resin than hLF.

A modified and improved procedure for the purification of hLF from milk is described in the Example under (II) Methods. Briefly, milk or a milk fraction, preferably skimmed milk, is contacted with a strong cation exchanger, preferably S Sepharose. The strong cation exchange resin containing the bound LF is washed with a buffer containing at least about 0.1 M salt, preferably NaCl, and then washed with a buffer containing at least about 0.4 M salt, preferably NaCl. After washing, the bound LF is eluted from the strong cation exchanger using a buffer containing at least 1.0 M salt, preferably NaCl, and concentrated and desalted to about 0.1 M of the salt. Optionally, at this stage the LF preparation is further microfiltrated and subjected to virus inactivation by adding a non-ionic surfactant, such as Tween 80 (at about 1% w/v), and a di- or trialkylphosphate, such as tri-n-butyl phosphate (at about 0.3% w/v), and incubating for at least 10 hours at a temperature between 15°C and 30°C, preferably about 25 ± 1°C. The (virus inactivated) LF sample is then loaded onto a Q Sepharose column serially connected with a Macroprep High S Support (MPHS, Biorad) column, both equilibrated in a buffer with about 0.1 M salt, preferably NaCl. The columns are washed with a buffer containing 0.1 M salt, preferably NaCl. The Q Sepharose column is then disconnected and the MPHS column is washed extensively with low salt buffer (e.g. 20mM sodium phosphate at pH 7.5). The MPHS column is stepwise eluted with first a buffer with about 0.4 M salt and then with a buffer with at least 0.5 M salt, preferably NaCl. Eluted fractions are tested for hLF (e.g., rhLF) content and endogenous LF (e.g. bLF) impurity. rhLF fractions free of endogenous LF (bLF) are pooled, concentrated, filtered over a 15 nm filter, concentrated, buffer exchanged to saline (0.9% NaCl), sterile filtrated (0.1 μm) and stored at < -65°C.

B. Production of variants and polypeptide fragments of LF

i. Polypeptides and variants

The present methods use intact lactoferrin and fragments and variants thereof. Some methods employ various polypeptides which include at least one or more arginines from the first cationic domain of hLF (residues 2-5 of SEQ ID NO:1), but which exclude the residues which make up the second cationic domain (residues 28-31 of SEQ ID NO:1). Thus, for example, some polypeptides comprise at least 6 but no more than 27 contiguous amino acids from the N-terminal segment of hLF (SEQ ID NO:1), wherein the N-terminus of the polypeptide is residue 1 of SEQ ID NO:1, *i.e.*, glycine.

Some methods employ polypeptides that include the first cationic domain but not the second cationic domain of hLF, and that also lack one or more residues from the N-terminus of hLF. For example, some methods of the invention use polypeptides comprising at least 6 amino acids but no more than 26 contiguous amino acids from hLF, wherein the N-terminus of the polypeptide is Arg^2 (for example, a hLF fragment lacking Gly^1). Some methods use polypeptides comprising at least 6 but no more than 25 contiguous amino acids from the N-terminal segment of hLF, wherein the N-terminus of the polypeptide is Arg^3 (for example, a hLF fragment lacking Gly^1Arg^2).

In some instances, the hLF polypeptide fragments just described are chosen so that cysteine residue 10 (see SEQ ID NO:1) is retained. In other cases, the polypeptide includes a cysteine at approximately the same location in the sequence. Such a cysteine can be used to dimerize one fragment with another polypeptide having a cysteine residue, such as another hLF fragment, for example. In some instances, dimerization can increase the activity of the polypeptide.

Some fragments include more than 6 contiguous amino acids from the N-terminus of hLF. For example, some polypeptides include 7, 8, 9, 10, 11 or 12 contiguous amino acids from hLF, for example. Some polypeptides include fewer than 27, 26 or 25 contiguous amino acids of hLF. Some polypeptides include less than 24, 22, 20, 18, 16, 14 or 12 contiguous amino acids from hLF, or any number of amino acids therebetween. Some polypeptides include no more than 19 contiguous amino acids from hLF. Some polypeptides include no more than 11 contiguous amino acids from hLF, for example, hLF(1-11), hLF(2-11) and hLF(3-11).

Some fragments of lactoferrin contain at least residues 1-90 of lactoferrin (designated N-terminal domain I). This domain has been show to bind specifically to phytohemagglutinin-stimulated peripheral blood human lymphocyte receptors (see

Rochard et al., Fed. Eur. Biochem. Soc. 1, 201-204 (1989). Some fragments contain at least residues 1-47. Such fragments have been reported to show bactericial activity (see Kiwata et al., Biochem. Biophys. Res. Commun. 245, 764-3 (1998)).

ii. Preparation

LF variants lacking one or more of the amino terminal arginine residues can be produced by a variety of methods. Preferred methods of production include (a) proteolytic cleavage of natural LF, (b) synthesis, or (c) recombinant expression, *e.g.*, mutagenesis of a LF gene followed by expression in cells or transgenic animals of the LF variant, with recombinant expression most preferred. Deletion of the residues of the second basic cluster is preferably carried out by *in vitro* mutagenesis.

a. Proteolysis

LF variants can be produced by cleavage of purified LF with a protease, preferably a serine protease and most preferably trypsin.

The tryptic digestion of purified natural LF can be carried out as follows: Five milligrams of native hLF are incubated with trypsin at an enzyme: substrate molar ratio of 1:8 at 37°C in PBS. Digestion is stopped after 1, 5, 25 min and 3 h by the addition of a 12-fold molar excess of SBTI and N-terminal integrity is monitored, for example by analytical Mono S chromatography (Van Berkel *et al.* (1995) *Biochem. J.* 312:107) and standard techniques such as SDS-PAGE, chromatography, and protein sequencing.

Following proteolysis, the LF variants can be separated from each other and from natural (*i.e.*, uncleaved) hLF (and other proteins, if present) by cationic exchange chromatography (e.g., Mono S; heparin), Hydrophobic Interaction Chromatography (HIC) or Cibracon Blue Sepharose chromatography. LF variants can be separated from uncleaved LF (and each other) by batch-wise incubation of recombinantly expressed LF or LF variants and S Sepharose for 4 h. The mixture is poured into a column and the LF eluted with 20 mM sodium phosphate, 0.5 M NaCl, pH 7.5. The S Sepharose eluate is diluted in 20 mM sodium phosphate, pH 7.5 (buffer A), applied on a Mono S HR 5/5 cation exchange column and eluted with a linear salt gradient of 0 to 0.5 M NaCl in 60 ml of buffer A at a flow rate of 0.5 ml/min. Natural hLF elutes at 0.7 M NaCl (Van Berkel *et al.* (1995) *Biochem. J.* 312:107) and hLF-5N elutes at about 0.33 M NaCl. The hLF-3N and hLF-2N species elute from Mono S at about 0.5 and about 0.6 M NaCl, respectively.

Certain polypeptides used in the present methods can also be prepared via a

reduction and proteolysis method. This approach begins with pepsin digestion of hLF according to known methods (see, *e.g.*, Bellamy, W. *et al.*, (1992) *Biochim. Biophys. Acta.* 1121:130; and Tomita, M. *et al.*, (1991) *J. Dairy Sci.* 74:4137). In order to break the disulfide bond between Cys 10 and Cys 46, the digested products are subsequently reduced and alkylated using standard reagents (*e.g.*, DTT or β-mercaptoethanol for reduction and iodoacetamine or 4-vinylpyridine for alkylation) according to known methods (see, *e.g.*, "Current Protocols in Protein Chemistry," Coligan, J.E., *et al.*, Eds. John Wiley and Sons, Inc). The order can be reversed so that the reduction and alkylation steps precede the pepsin digestion step. After digestion, reduction and alkylation, N-terminal peptides can be isolated from the digestion mixture by standard chromatographic methods, including for example, cation exchange, gel filtration, HIC or RP-HPLC. Further useful hLF variants can be obtained by proteolytic cleavage of purified intact hLF using one or more proteases, such as bromelain, cathepsin B, cathepsin D, cathepsin G, chymotrypsin, clostripain, elastase, endoproteinase-Arg-C, endoproteinase-Asp-N, endoproteinase-Glu-C, endoproteinase-Lys-C, Factor Xa, papain, pepsin, proteinase K, subtilisin, thermolysin and trypsin. The above proteases are commercially available.

Intact lactoferrin can also be converted to fragments by a variety of mechanisms in vivo. For example, lactoferrin can be degraded by release of elastase (a serine protease) as a result of neutrophil degranulation (Nuijens et al., J. Lab. Clin. Med. 119, 159-168 (1992). Lactoferrin can also be degraded by bacterial derived protease (see Britigan et al., J. Clin. Invest. 88, 1092-102 (1991), by membrane bound proteases (see Birgens et al. Eur. J. Haematol. 45, 31-35 (1990) and as a result of pepsin degradation in the stomach (see Kuwata et al., Biochem. Biphys. Res. Comm. 245, 764-73 (1998). Fragments of the type produced by in vivo degradation of intact lactoferrin, for example, at sites of inflammation or infection, are also particularly useful agents for direct administration. Such fragments can be produced in vitro by vitro proteolytic cleavage of intact hLF, using one or more of the above-mentioned commercially available endoproteases. Preferentially, the in vitro produced hLF derived peptides are purified using conventional means before formulating as a pharmaceutical composition for administration..

b. Synthesis

Fragments of lactoferrin can be synthesized by the well-known Merrifield solid-phase synthesis method in which amino acids are sequentially added to a growing chain. See Merrifield (1963), *J. Am. Chem. Soc.* 85:2149-2156; and Atherton *et al.*, "Solid Phase

Peptide Synthesis," IRL Press, London, (1989). Automatic peptide synthesizers are commercially available from numerous suppliers, such as Applied Biosystems, Foster City, California.

c. Recombinant techniques

Alternatively, intact lactoferrin and fragments and variants thereof can be prepared using recombinant techniques in which a nucleotide sequence encoding the polypeptide of interest is expressed in cultured cells such as described in Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York (1987) and in Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, 2nd ed., vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989) both of which are incorporated herein by reference in their entirety. Also see, Kunkel (1985) *Proc. Natl. Acad. Sci.* 82:488 (describing site directed mutagenesis) and Roberts *et al.* (1987) *Nature* 328:731-734 or Wells, J.A., *et al.* (1985) *Gene* 34:315 (describing cassette mutagenesis).

Typically, nucleic acids encoding the desired polypeptides are used in expression vectors. The phrase "expression vector" generally refers to nucleotide sequences that are capable of affecting expression of a gene in hosts compatible with such sequences. These expression vectors typically include at least suitable promoter sequences and optionally, transcription termination signals. Additional factors necessary or helpful in effecting expression can also be used as described herein. DNA encoding a polypeptide is incorporated into DNA constructs capable of introduction into and expression in an *in vitro* cell culture. Specifically, DNA constructs are suitable for replication in a prokaryotic host, such as bacteria, *e.g.*, *E. coli*, or can be introduced into a cultured mammalian, plant, insect, e.g., Sf9, yeast, fungi or other eukaryotic cell lines.

DNA constructs prepared for introduction into a particular host typically include a replication system recognized by the host, the intended DNA segment encoding the desired polypeptide, and transcriptional and translational initiation and termination regulatory sequences operably linked to the polypeptide encoding segment. A DNA segment is "operably linked" when it is placed into a functional relationship with another DNA segment. For example, a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence. DNA for a signal sequence is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide. Generally, DNA sequences that are

operably linked are contiguous, and, in the case of a signal sequence, both contiguous and in reading phase. However, enhancers need not be contiguous with the coding sequences whose transcription they control. Linking is accomplished by ligation at convenient restriction sites or at adapters or linkers inserted in lieu thereof.

The selection of an appropriate promoter sequence generally depends upon the host cell selected for the expression of the DNA segment. Examples of suitable promoter sequences include prokaryotic, and eukaryotic promoters well-known in the art. *See, e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2d ed.), vols. 1-3, Cold Spring Harbor Laboratory (1989). The transcriptional regulatory sequences typically include a heterologous enhancer or promoter which is recognized by the host. The selection of an appropriate promoter depends upon the host, but promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters are known and available. *See* Sambrook *et al.*, *supra*. Expression vectors include the replication system and transcriptional and translational regulatory sequences together with the insertion site for the polypeptide encoding segment can be employed. Examples of workable combinations of cell lines and expression vectors are described in Sambrook *et al.*, *supra*, and in Metzger *et al.* (1988) *Nature* 334:31-36. For example, suitable expression vectors can be expressed in, *e.g.*, insect cells, *e.g.,* Sf9 cells, mammalian cells, *e.g.*, CHO cells and bacterial cells, *e.g.*, *E. coli*.

In vitro mutagenesis and expression of mutant proteins are described generally in Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York (1987) and in Sambrook *et al.*, Molecular Cloning - A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989, both of which are incorporated herein by reference in their entirety and for all purposes. Also see, Kunkel (1985) *Proc. Natl. Acad. Sci.* 82:488 (describing site directed mutagenesis) and Roberts *et al.* (1987) *Nature* 328:731-734) or (Wells, J.A., *et al.* (1985) *Gene* 34:315 (describing cassette mutagenesis).

Another method for preparing polypeptides is to employ an *in vitro* transcription/translation system. DNA encoding a polypeptide is cloned into an expression vector as described *supra*. The expression vector is then transcribed and translated *in vitro*. The translation product can be used directly or first purified. Polypeptides resulting from *in vitro* translation typically do not contain the post-translation modifications present on polypeptides synthesized *in vivo*. Methods for synthesis of polypeptides by *in vitro* translation are described by, for example, Berger &

Kimmel, *Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques*, Academic Press, Inc., San Diego, CA, 1987 (incorporated herein by reference in its entirety).

C. Transgenic animals

In certain instances, hLF or hLF polypeptides or variants are produced by expression in transgenic animals (*i.e.*, non-human animals containing an exogenous DNA sequence in the genome of germ-line and somatic cells introduced by way of human intervention) such as bovines, goats, rabbits, sheep, pigs or mice. Methods for production of recombinant polypeptides by transgenic non-human species are known in the art and are described, for example, in U.S. Patent Nos. 5,304,489; 5,633,076; and 5,565,362 which are incorporated herein by reference in their entirety, as well as in PCT publications PCT/US93/05724 and PCT/US95/09580, both of which are incorporated herein by reference in their entirety. An advantage of the transgenic animals is the isolation of hLF, variants and polypeptides of interest in large amounts, especially by economical purification methods. For example, the production of transgenic bovine species containing a transgene encoding a human LF polypeptide targeted for expression in mammary secreting cells is described in WO 91/08216, incorporated herein by reference in its entirety. When LF variants are produced in transgenic bovines the human protein typically is separated from the bovine milk proteins (*e.g.*, whey proteins, caseins, bovine LF, IgA, albumin, lysozyme, β-lactoglobulin) before use (*e.g.*, administration to patients). Alternatively, use can be made of whole or partially purified bovine milk containing the desired hLF protein, variant or polypeptide.

D. Alternative Methods for Neutralizing hLF Basic Clusters

Although deletion of the residues in the first or second basic cluster of hLF is a preferred method for generating a hLF with changed physiological properties, other methods for neutralizing one or both basic clusters exist. For example, the first basic cluster can be neutralized by incubating hLF with ligands such as heparin, which binds at the first cluster and inhibits binding of LF to the 105kd LF receptor, LPS, hLZ, and other molecule for which binding is first-cluster dependent.

A preferred method for neutralizing the first basic cluster is to incubate hLF with a monoclonal antibody that binds at the amino terminus and prevents binding between the

first basic cluster and a target molecule (*e.g.*, heparin). Methods for producing monoclonal antibodies are well known (see, e.g., Goding *et al.*, Monoclonal Antibodies: Principles and Practice (2d ed.) Acad. Press, N.Y., and Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York , 1988). Use of human or humanized monoclonal antibodies are most preferred because this reduces the possibility of an antigenic response following administration to a patient (see *e.g.*, U.S. Patent Nos. 5,569,825 and 5,585,089). Antigen-binding fragments of monoclonal antibodies, *e.g.*, Fab, Fab' F(ab')2, Fabc and Fv fragments, are similarly useful. Antibodies or antibody fragments can also be used for binding to the second basic cluster and preventing second-cluster dependent binding.

IV. Properties of lactoferrin and lactoferrin variants

A. Binding to heparin, Lipid A, DNA and human lysozyme

Natural hLF binds to heparin, Lipid A, DNA and hLZ. LF variants lacking one, two or three arginines of the first basic cluster show a strong decrease in reactivity for each of these four ligands, and binding is undetectable in standard assays (*e.g.*, solid phase ligand binding assays) when all four of the amino-terminal arginine residues are deleted.

The binding properties of natural LF and the LF variants can be measured in standard binding assays (see next section) and expressed in terms of *reactivity* where the level of binding of natural LF to a ligand is defined as 100% reactivity. The reactivity of the LF variants with the natural LF ligands heparin, DNA, Lipid A and hLZ is typically less than 80%, more usually less than 60%, often less than 15%. For some LF variants (*e.g.*, those with a deletion of all four arginine residues from the first basic cluster) reactivity is undetectable.

B. Binding assays

Assays suitable for measuring ligand-receptor interactions, such as the binding by natural LF and LF variants to heparin, lipid A, DNA, and hLZ, include assays solid-phase ligand binding assays and competitive solid-phase binding assays (see, *e.g.*, Mann *et al.* (1994) *J. Biol. Chem.* 269:23661-67). In some methods, the solid-phase binding assays l measure binding by LF variants and natural LF. Typically, binding of the LF receptor by hLF or a hLF variant results in activation of the LF receptor. Methods for assaying receptor activation are known, for example, the resulting intracellular calcium shift can be measured (*see, e.g.*, Misra *et al.* (1994) *J. Biol. Chem.* 269:18303-306).

C. Specific binding to cell membrane associated receptors

Some of the biological activities of hLF are linked to its ability to strongly chelate iron, whereas other activities relate to the interaction of hLf with target cells, including intestinal cells (Hu *et al.* (1990) *Biochemistry* 29, 535-541; Kawakam *et al.* (1991) *Am. J. Physiol.* 261, G841-G846; Mikogami *et al.* (1994) *Am. J. Physiol.* 267, G308-G31), mammary gland epithelial cells (Rochard *et al.* (1992) *Anticancer Res.* 1, 2047-2052), hepatocytes (Regoeczi *et al.* (1985) *Am. J. Physiol.* 248, G8-G14; MacAbee *et al*, (1991) *J. Biol. Chem.* 226, 23624-23631; Ziere *et al*, (1992) *J. Biol. Chem.* 267, 11229-11235), monocytes (Ismail *et al*, (1993) *J. Biol. Chem.* 268, 21618-21625), activated lymphocytes (Mazurier *et al*, (1989) *Eur. J. Biochem.* 179, 481-487) and platelets (Leveugle *et al*, (1993) *Eur. J. Biochem.*, 213,1205-1211) each of which is incorporated by reference in their entirely and for all purposes.

LF binds to cell surfaces through two classes of LF binding sites: relatively low affinity sites which are cell surface sulfated molecules (*e.g.*, cell surface proteoglycans or glycosaminoglycans) and high affinity receptors. Binding to the low affinity sites is mediated by the first cluster of basic arginine residues, and deletion (or neutralization) of one or more of these residues reduces or eliminates binding to the low affinity sites. Thus, an hLF variant typically binds a high affinity LF receptor with an affinity of at least about 10 nM, usually between about 10 nM and about 40 nM. Cell binding assays are well known and are described in, *e.g.*, Mazurier (1989) *Eur. J. Biochem.* 179:481-87. In contrast, deletion of one or more of the amino-terminal arginine residues does not reduce or abolish binding to the high affinity LF receptor.

High affinity LF binding sites have been found on activated lymphocytes, mammary gland epithelial cells, platelets, monocytes, macrophages, intestinal cells, and hepatocytes and are thought to exist on other cell types as well. A 105 kD specific hLF receptor has been characterized in activated lymphocytes (Mazurier *et al.* (1989) *Eur. J. Biochem.* 179, 481-487), the Jurkat T-cell line (Bi *et al.* (1994) *Eur. J. Cell Biol.* 65, 164-171; Bi *et al.* (1996) *Eur. J. Cell Biol.* 69, 288-296) and platelets (Leveugle *et al.* (1993) *Eur. J. Biochem.*, 213,1205-1211).

Binding of LF to the 105 kD receptor has been shown to inhibit platelet aggregation and is likely involved in the growth factor and/or differentiation activities of hLF. This receptor has been localized in human lymphoblastic T-cells (*i.e.*, Jurkat cells, Pawelec *et al.*(1982) *Eur. J. Immuno.* 12:387-92) to the cell surface in coated pits vesicles as well as

in intracellular vesicles. Internalization of hLF by Jurkat cells has been demonstrated. Jurkat cells can be obtained from the American Tissue Type Collection American Type Culture Collection [ATCC] located at 12301 Parklawn Dr., Rockville, Maryland, USA 20852. LF binds to the lymphocyte (Jurkat cell) high affinity receptor with a kD of approximately 40 nM.

The 105 kD receptor can be identified by immunological methods. For example, a specific rabbit anti-105 kD receptor polyclonal antibody has been described. This, or a similar polyclonal antibody, or an anti-105 kD receptor monoclonal antibody, can be used to identify the receptor on other cell types. For example, the polyclonal antibody referred to *supra* has been found to bind to epithelial cells from non-malignant human breast, benign mastopathies and breast carcinomas (Rochard *et al.* (1992) *Anticancer Research* 12: 2047-52). Alternatively, the 105 kD receptor can be identified by ligand blotting (see, *e.g.*, Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York) using labeled hLF (or amino-terminally deleted hLF) and membrane protein preparations of cells.

A specific hLF receptor has been isolated from intestinal brush border membranes and has a reported Mr of 110 (Kawaskami and Lonnerdal (1991) *Am. J. Physiol.* 261:G841-46). It is likely, but has not yet been demonstrated, that this receptor is the same as, or closely related to, the 105 kD receptor.

In hepatocytes, LF binds to a chylomicron remnant receptor or the Low density lipoprotein Receptor-related Protein (LRP) present on the cell surface. LF inhibits uptake of beta-VLDL containing chylomicron remnants. LF binding to murine peritoneal macrophages apparently occurs via the LRP, a member of the structurally related cell surface receptor family that mediates endocytosis of lipoproteins and other plasma proteins. The nature of hLF binding to monocytes and macrophages is incompletely characterized, although it is appears to be mediated, at least in part, a member of the LRP/chylomicron remnant receptor (Misra *et al.* (1994) *J. Biol. Chem.* 269:18303-306).

V. Pharmaceutical applications

A. Indications

High dosage parenteral administration of lactoferrin or a pharmaceutical composition containing the same can be used in a variety of therapeutic and prophylactic applications. Furthermore, it will be clear that lactoferrin, and the fragments and variants thereof as described herein may be used in the preparation of medicaments, such as the

pharmaceutical compositions described herein, for the treatment and/or prophylaxis of the indications described herein.

For example, the methods are useful in treating various microbial infections such as bacterial infections. The methods are also useful in providing anti-inflammatory, anti-viral and anti-infective activities, as well a pro- and anti-coagulant effects, modulation of complement activation, inhibition of LPS mediated activation of neutrophils, regulation of transcription, growth promotion of intestinal epithelial cells, inhibition of hydroxyl-radical formation, and a role in intestinal iron uptake and excretion. Other properties and biological activities of LF are described in Nuijens *et al.* (1996) *J. Mammary Gland Biology and Neoplasia* 1:3, 283-293, which is incorporated herein by reference in its entirety and for all purposes.

hLF variants and neutralized LF have generally the same activities and uses as natural LF, except that deletion of the arginines of the first basic cluster results in reduction in binding to heparin, Lipid A, DNA, lysozyme, and cell surface sulfated molecules. Thus, some LF fragments can be administered to a patient to effect certain LF-mediated physiological changes (*e.g.*, regulation of cytokines) without causing other physiological consequences of LF administration (*e.g.*, neutralization of heparin by binding). Some lactoferrin fragments have various activities associated with the first cationic cluster of hLF. Such polypeptides are especially useful in selectively triggering responses involving the first cationic domain while avoiding the activation of responses associated with the binding of the second cationic domain and/or activities related to the iron binding activities of hLF. The neutralized hLF and hLF variants have a variety of advantageous properties. For example, hLF variants lacking the first basic cluster are particularly useful for initiating hLF-receptor-mediated immune and inflammatory responses (*e.g.* , reducing cytokine release, activation of natural killer cells, and anti-tumor effects), efficient receptor mediated delivery of nutritional iron, and other biological effects.

Some lactoferrin fragments can bind and neutralize heparin and LPS, lipid A, and DNA and hLZ. Some lactoferrin fragments can also bind to various target cells. LF binds to cell surfaces through two classes of LF binding sites: relatively low affinity sites which are cell surface sulfated molecules (*e.g.,* cell surface proteoglycans or glycosaminoglycans) and high affinity receptors. Since binding of LF to the low affinity sites involves the first cationic domain, some lactoferrin fragments can selectively bind to the low affinity sites without activating the LF high affinity receptors. Thus, for example,

some lactoferrin fragments are useful in neutralizing heparin or LPS without activating the LF high affinity receptor. Some lactoferrin fragments can neutralize the anticoagulant activity of heparin (including low molecular weight heparin). By neutralizing bacterial LPSs, some lactoferrin fragments can reduce the inflammatory response associated with these compounds. Such lactoferrin fragments are also used to inhibit viral entry into cells. Cell binding assays are well-known and are described in, *e.g.,* Mazurier (1989) *Eur. J. Biochem.* 179:481-87.

Cells with which lactoferrin, its fragments and variants can interact with include, intestinal cells (Hu *et al.* (1990) *Biochemistry* 29:535-541; Kawakam *et al.* (1991) *Am. J. Physiol.* 261:G841-G846; Mikogami *et al.* (1994) *Am. J. Physiol.* 267:G308-G31), mammary gland epithelial cells (Rochard *et al.* (1992) *Anticancer Res.* 1:2047-2052), hepatocytes (Regoeczi *et al.* (1985) *Am. J. Physiol.* 248:G8-G14; MacAbee *et al.* (1991) *J. Biol. Chem.* 226:23624-23631; Ziere *et al.* (1992) *J. Biol. Chem.* 267:11229-11235), monocytes (Ismail *et al.* (1993) *J. Biol. Chem.* 268:21618-21625), activated lymphocytes (Mazurier *et al.* (1989) *Eur. J. Biochem.* 179, 481-487) and platelets (Leveugle *et al.* (1993) *Eur. J. Biochem.*, 213:1205-1211), each of which is incorporated by reference in their entirety for all purposes.

Intact lactoferrin and certain fragments and variants can also be used to inhibit entry into a cell of viruses, for example, cytomegalovirus (CMV), human immunodeficiency viruses (HIV) or herpes simplex virus 1 (HSV1) viruses. While not intending to be limited to this particular explanation, the antiviral action is thought to be mediated by interaction of hLF with cell surface proteoglycans (*e.g.*, heparin) employed by viral particles for cell entry, and/or by the stimulation of natural killer cells.

Therapeutic indications include use in therapy or prophylaxis of infection, including local infection, large scale (bacterial) infection, blood-borne infection (sepsis), as well as inflammation resulting from an infection or non-infectious inflammatory diseases (*e.g.*, chronic inflammatory disease of the ileum or colon). The compositions can also be used to prepare or treat organ transplant recipients or other immunosuppressed individuals (*e.g.*, AIDS patients) against the effects of infections.

The pharmaceutical compositions are effective in treating a variety of microbial infections, such as various viral and bacterial infections. For example, the compositions are effective in treating Gram-negative and Gram-positive bacteria. More specifically, some examples of pathogenic bacteria causing infections treatable by methods of the invention include: *Listeria, Escherichia, chlamydia, rickettsial* bacteria, *mycobacteria,*

staphylococci, streptocci, pneumonococci, meningococci and conococci, Klebsiella, proteus, serratia, pseudomonas, Legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and *Lymes* disease bacteria.

Some examples of pathogenic viruses causing infections treatable by methods of the invention include: *hepatitis* (A, B, or C), *herpes* virus (e.g., VZV, HSV-I, HAV-6, HSV-II, and CMV, *Epstein Barr* virus), *adenovirus, influenza* virus, *flaviviruses, echovirus, rhinovirus, coxsackie* virus, *coronavirus*, respiratory syncytial virus (RSV), *mumps* virus, *rotavirus, measles* virus, *rubella* virus, *parvovirus*, vaccinia virus, HTLV virus, *dengue* virus, *papillomavirus, molluscum* virus, *poliovirus, rabies* virus, JC virus, *arboviral encephalitis* virus, and human immunodeficiency virus (HIV virus; *e.g.*, type I and II).

Some examples of pathogenic fungi causing infections treatable by methods of the invention include: *Candida* (*e.g., albicans, krusei, glabrata, tropicalis*), *Cryptococcus neoformans, Aspergillus* (*e.g., fumigatus, niger*), Genus *Mucorales* (Mucor, Absidia, Rhizopus), *Sporothrix schenkii, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis* and *Histoplasma capsulatum*. Some examples of pathogenic parasites causing infections treatable by methods of the invention include: *Entamoeba histolytica, Balantidium coli, Naegleria, Fowleri, Acanthamoeba sp., Giardia lambia, Cryptosporidium sp., Pneumocystis carinii, Plasmodium vivax, Babesia microti, Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani, Toxoplasma gondii* and *Plasmodium falciparis*.

Lactoferrin is particularly useful for the treatment of inflammatory diseases. This can occur, as noted above, through neutralization of bacterial LPSs (see also Lee et al., Infect. Immun 66, 1421-6 (1998), as well as through a reduction in cytokine production and neutrophil degranulation. Thus, in another aspect, the invention provides methods in which hLF or an LF variant is administered to a patient to reduce inflammation, for example in chronic inflammatory bowel disease (*e.g.*, Crohn's disease and ulcerative colitis). Administration of hLF and hLF variants is useful for reducing reperfusion injury in a patient after myocardial infarction. hLF can be administered to neutralize bacterial LPS. The LPS binds through the first basic cluster, and is cleared from circulation via the second basic cluster.

The invention also provides methods in which lactoferrin is administered to a patient to inhibit myelopoieses and reduce production of GM-CSF.

Intact hLF, hLF variants and neutralized hLF are also useful for reducing or inhibiting release of a cytokine, such as IL-1, IL-2 or TNF-alpha, from LF-receptor

bearing cells in a patient, by administering LF or a LF variant. LF has been shown to reduce the release of cytokines, *e.g.*, IL-1, IL-2, and TNF-alpha from cells, and inhibit proliferation in mixed lymphocyte cultures (Chierici *et al.* (1994) *Acta Pediatr Suppl* 402:83-89). Suppression of IL-1 and TNF-alpha release from monocytes in response to LPS by hLF and variants is expected to down regulate recruitment and activation of neutrophils at inflammation sites (*see, e.g.*, Lonnerdal *et al.* (1995) *Ann Rev Nutr* 15:93-110). The suppressive effects of LF are thought to be mediated through the binding of LF to monocyte lactoferrin-receptors (Miyazawa *et al.* (1991) *J. Immunol.* 146:723-729), and can be responsible for the prophylactic effect of LF in mice injected intravenously with a lethal dose of *E. coli* (Sanchez *et al.* (1992) *Arch Dis Child.* 67:657-661) since LPS-mediated TNF responses in mice were attenuated by prior administration of LF (Lonnerdal *et al.*, *supra*). Methods for measuring cytokine release are well known (*e.g.*, ELISA). A reagent can be said to reduce or inhibit release of a cytokine from a cell when the level of cytokine release in the presence of the reagent is less than about 90%, more often less than about 70%, and most often less than about 50% of the levels released in the absence of the reagent under the conditions of the assay.

Intact lactoferrin, fragments and variants thereof can be administered to a patient to reduce TNF-alpha-mediated neutrophil degranulation. Neutrophils have been implicated as important mediators in both generalized and local inflammatory reactions, including sepsis, rheumatoid arthritis and ulcerative colitis. For example, clinical studies using anti-TNF monoclonal antibodies indicate that TNF, and likely the TNF-mediated activation of neutrophils, plays an important role in the pathophysiology of rheumatoid arthritis and ulcerative colitis.

Intact lactoferrin, fragments and variants thereof are useful for stimulating natural killer (NK) cells in the patient. Because hLF and LF variants cause stimulation of natural killer (NK) cells, the LF variants are useful for combating the targets of NK cells, *e.g.*, tumors, viruses and intracellular pathogens. Stimulation of natural killer (NK) cells by LF has been shown *in vitro* (Shau *et al.*, 1992, *J. Leukoc. Biol.* 51:343-349) and *in vivo* (Bezault *et al.*, 1994, *Cancer Res.* 54:2310-2312). NK cells can be stimulated in a patient by administering to the patient a composition comprising a hLF variant and a pharmaceutical excipient. hLF and LF variants can also be administered to a patient to inhibit growth of a solid tumor. A single intraperitoneal injection of LF inhibited growth of solid tumors induced by subcutaneous injection of syngeneic fibroblast-derived tumor

cell lines in mice (Bezault *et al.*, *supra*). LF variants are thus useful for stimulation of NK cells without neutralization of heparin or other undesirable effects.

In other methods, intact lactoferrin, fragments and variants thereof are used to deliver iron to an LF-receptor-bearing cell in a patient by administering to the patient a composition of hLF or a LF fragment or variant which is at least about 95% saturated with iron. Administration of these compounds are beneficial, for example, in treatment of anemia or iron storage diseases. LF- or LF-variant bound iron is delivered to a cell when the polypeptide-iron complex binds to a cell receptor and is internalized by the cell. Thus the compositions disclosed herein are suitable for use in baby formula as well as for administration to patients with disturbances in iron metabolism (*e.g.*, ferriprive anemia and iron storage diseases, and iron deficiency anemia of premature infants). LF variants can be saturated with iron following the procedure described in van Berkel *et al.*, 1995, *Biochem J.* 312, 107-114. A LF variant is at least 3% saturated with iron, more usually 80% saturated, most often at least 95% saturated and often more than 99% saturated. LF variants lacking the first basic cluster, or both the first and second basic clusters are particularly useful when the iron binding activities of LF are desired and when the activities mediated by basic clusters 1 and 2 (*e.g.*, heparin binding, high affinity receptor interaction) are not desired.

B. Pharmaceutical Compositions

Intact lactoferrin, fragments and variants can be used as pharmaceutical, food additives, nutritional supplements, and the like. Such pharmaceutical compositions are usually administered parenterally, preferably intravenously. Intradermal, topical, enteral or intramuscular administration is also possible in some circumstances. Oral administration can also be used, optionally but not necessarily, in conjunction with parenteral administration.

The compositions containing the compounds can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications, particularly symptoms associated with a microbial infection, or otherwise prevent, hinder, retard, or reverse the progression of disease or infection or any other undesirable symptoms in any way whatsoever. An amount adequate to accomplish this is defined as "therapeutically effective amount or dose." Such effective dosage depends on the nature

and severity of the disease or condition, and on the general state of the patient's health, but is usually be between 10 and 100 mg of intact human lactoferrin per kilogram of body weight. In some methods, the dosage is at least 20, 30, 40, 50, or 60 mg/kg for intact lactoferrin. Fragments or variants having different molecular weights than intact lactoferrin can be administered in equimolar ratio to the above dosages or can be administered in higher dosages up to the mg/kg dosage given for intact lactoferrin. Dosages of 10, 20, 30, 40, 50, 60 and 100 mg/kg intact hLF are equal to 130, 260, 390, 520, 650, 780 and 1300 nmol/kg respectively (hLF Mr 77,000). Thus, for example, if a peptide/fragment has Mr 1500, a dosage of 0.19 mg/kg is in equimolar ratio to a dosage of 10 mg/kg intact human lactoferrin. The concentration of the polypeptide in the pharmaceutical composition can vary widely, *i.e.,* from less than about 0.1% by weight, usually being at least about 1% by weight, to as much as 20% by weight or more.

In prophylactic applications, intact lactoferrin, fragments or variants thereof, or pharmaceutical compositions containing the same are administered to a patient susceptible to or otherwise at risk of a particular disease or infection. Such an amount is defined to be a "prophylactically effective" amount or dose. In this use, the precise amounts again depends on the patient's state of health and weight. Typically, the dose is between 10 and 100 mg of intact human lactoferrin per kilogram of body weight. In some methods, the dosage of intact human lactoferrin is at least 20, 30, 40, 50, or 60 mg/kg. Dosages for fragments and variants can be adjusted to be in equimolar ratio with dosages specified for intact human lactoferrin as described above. Alternatively, fragments and variants can be administered at dosages up to the mg/kg dosage indicated for intact human lactoferrin.

In both therapeutic and prophylactic methods, lactoferrin, fragments and variants thereof can be administered as a single dosage or as a series of dosages over time. In some methods, a dosage of amount described above is administered at least daily for a period of a week, a month or a year. In other methods, a dosage of the above amount is administered at least twice weekly or weekly for a period of a month, a year, the duration of a disease or for the life of the patient.

Compositions prepared for intravenous administration typically contain 100 to 500 ml of sterile 0.9% NaCl or 5% glucose optionally supplemented with a 20% albumin solution and 100 to 500 mg of intact lactoferrin. Sterile Ringer's solution can also be used. A typical pharmaceutical composition for intramuscular injection would be made up to contain, for example, 1 ml of sterile buffered water and 10 to 100 mg of intact lactoferrin

Methods for preparing parenterally administrable compositions among others are well-known in the art and described in more detail in various sources, including, for example, *Remington's Pharmaceutical Science*, Mack Publishing, Philadelphia, PA, 17th ed., (1985) and Langer, *Science* 249:1527-1533 (1990) (both incorporated by reference in their entirety for all purposes).

The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers of diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Typically the hLF, or hLF polypeptide fragments or variants are administered along with a pharmaceutical excipient or carrier comprising any compatible, non-toxic substance suitable to deliver the polypeptides to the patient. Examples of such diluents are sterile water, distilled water, buffered water, physiological saline, PBS, Ringer's solution, dextrose solution, Hank's solution., alcohol, fats, waxes, and inert solids and can be used as the excipient or carrier. In addition, the pharmaceutical composition or formulation can also include other pharmaceutically acceptable carriers, adjuvants, or non-toxic, non-therapeutic, non-immunogenic stabilizers, excipients, buffering agents, dispersing agents, and the like. The compositions can also include additional substances to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents, wetting agents, detergents and the like. Because of the ability of hLF to bind iron, in some instances it can be beneficial to include iron in the pharmaceutical composition. The concentration of the polypeptide in the pharmaceutical composition can vary widely, *i.e.*, from less than about 0.1% by weight, usually being at least about 1% by weight to as much as 20% by weight or more.

The composition can also include any of a variety of stabilizing agents, such as an antioxidant for example. Moreover, the polypeptides can be complexed with various well-known compounds that enhance the *in vivo* stability of the polypeptide, or otherwise enhance its pharmacological properties (*e.g.*, increase the half-life of the polypeptide, reduce its toxicity, enhance solubility or uptake). Examples of such modifications or complexing agents include the production of sulfate, gluconate, citrate, phosphate and the like. The polypeptides of the composition can also be complexed with molecules that enhance their *in vivo* attributes. A list of such molecules, provided by way of example and not limitation, includes carbohydrates, polyamines, amino acids, other peptides, ions (*e.g.*, sodium, potassium, calcium, magnesium, manganese), and lipids.

For oral administration, optionally in conjunction with parenteral administration, the active ingredient can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. The active component(s) can be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. Examples of additional inactive ingredients that can be added to provide desirable color, taste, stability, buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide, and edible white ink. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient acceptance. Pharmaceutical compositions can be administered with a foodstuff, typically milk, *e.g.*, bovine milk. This mode of administration have advantages when the LF/variant is produced by expression in a transgenic animal such as a transgenic bovine, goat, or rabbit. The production of LF in transgenic bovine milk is desirable since it provides a matrix wherein little or no purification is necessary for human consumption.

If desired, for example in the treatment of infections or disorders of the digestive tract or even for general oral administration of the compositions, it is possible to formulate solid or liquid formulations in an enteric-coated or otherwise protected form. In the case of liquid formulations, the formulation can be mixed or simply coadministered with a Protestant, such as a liquid mixture of medium chain triglycerides, or the formulation can be filled into enteric capsules (*e.g.,* of soft or hard gelatin, which are themselves optionally additionally enteric coated). Alternatively, solid formulations comprising the polypeptide can be coated with enteric materials to form tablets. The thickness of enteric coating on tablets or capsules can be, for example, from 0.5 to 4 microns in thickness. The enteric coating can comprise any of the enteric materials conventionally utilized in orally administrable pharmaceutical formulations. Suitable enteric coating materials are known, for example, from *Remington's Pharmaceutical Sciences*, Mace Publishing Company, Philadelphia, 17th ed. (1985); and *Hagars*

Handbuch der Pharmazeutischen Praxie, Springer Verlag, 4th ed., Vol. 7a (1971), both of which are incorporated herein by reference in their entirety.

Another delivery option involves loading the composition into lipid-associated structures (*e.g.*, liposomes, or other lipidic complexes) which can enhance the pharmaceutical characteristics of the polypeptide component of the composition. The complex containing the composition can subsequently be targeted to specific target cells by the incorporation of appropriate targeting molecules (*e.g.*, specific antibodies or receptors). It is also possible to directly complex the polypeptide with a targeting agent.

The particular form of the composition varies with the intended mode of administration and therapeutic application. Typically, however, the composition includes polypeptides comprising full-length LF, fragments of LF, and/or variants of LF, and a pharmaceutically acceptable excipient. The polypeptide included in the composition has a defined length, can consist of one or more arginine residues at the N-terminus and have antimicrobial activity (*e.g.*, is effective in killing viruses or bacteria). In certain compositions, the polypeptide includes a contiguous segment of up to 27 amino acids and the N-terminal amino acids of the polypeptide consists of the residues XRR (where X is any amino acid and R is arginine. Some compositions include polypeptide no longer than 26 amino acids and the N-terminal amino acids are RR. Some compositions include a polypeptide no longer than 25 amino acids and the N-terminus is R (*i.e.*, Arg). In other instances, the polypeptide is shorter, such as 5, 10, 15, 20 or 25 amino acids long, or any length therebetween. The polypeptide is even smaller in other compositions. For instance, the polypeptide can simply consist of the N-terminal XRR or RR residues. The polypeptides used in the pharmaceutical compositions can also include any of the polypeptides described above.

While the N-terminus of the polypeptide consists of the amino acids XRR, RR or R, the remaining contiguous amino acid sequence of the polypeptide sequence can vary so long as the polypeptide has antimicrobial activity. For example, the remaining contiguous sequence can consist of a contiguous amino acid sequence from hLF, especially the sequence beginning after Arg^3. Thus, the polypeptide can be hLF(1-11), hLF(2-11) or hLF(3-11). When the polypeptide used in the pharmaceutical composition consists of a contiguous amino acid sequence from the N-terminal segment of hLF, the amino acid sequence can include sequences wherein a small number (*e.g.*, one, two or three) amino acids are inserted or removed from the hLF sequence. Alternatively, the polypeptide includes contiguous sequences from hLF, wherein one or more of the amino acids has

been chemically modified.

Particularly when the compositions are to be used *in vivo*, the components used to formulate the pharmaceutical compositions are preferably of high purity and are substantially free of potentially harmful contaminants (*e.g.,* at least National Food (NF) grade, generally at least analytical grade, and more typically at least pharmaceutical grade. To the extent that a given compound must be synthesized prior to use, the resulting product is typically substantially free of any potentially toxic agents, particularly any endotoxins, which may be present during the synthesis or purification process. Compositions for parental administration are also sterile, substantially isotonic and made under GMP conditions.

VI. Monitoring

In some methods, a patient is monitored after administration of lactoferrin, fragments or variants thereof to determine beneficial effects and/or side effects responsive to administration of lactoferrin. Monitoring can be for subjective symptoms, such as headache or nausea reported by the patient, physiological characteristics, such as temperature, presence of rash, and blood pressure, or by biochemical analysis of tissue samples from the patients. Side effects are deemed insubstantial if they do not prevent repeated administration of the same drug to the patient. Typically, insubstantial side effects resolve spontaneously within 24 hr, and preferably within 12 hr. Typically, insubstantial side effects do not recur with greater severity on repeated administration of the drug.

EXAMPLES

I. Introduction

During coronary artery bypass surgery (CABG) operations heparin in doses of 300-500 U/kg is used to prevent the blood from coagulating and to prevent thrombosis. After the patient's normal perfusion has been restored (i.e. after termination of the operation), the action of heparin needs to be terminated quickly to prevent hemorrhaging. The action of heparin can be terminated by recombinant rhLF, since rhLF binds to heparin and can prevent the anticoagulant effect of heparin.

This Example describes a clinical study in healthy male volunteers that examined the safety of four different dose levels of intravenous rhLF alone and / or in combination with heparin. In addition, the effect of rhLF on the neutralization of heparin and the pharmacokinetics of rhLF was investigated. The volunteers were also monitored for any signs of adverse reactions due to heparin or rhLF.

II. Methods

Materials

The products administered as single intravenous doses were recombinant human lactoferrin; heparin; placebo recombinant human lactoferrin; placebo heparin. The study medication was manufactured from transgenic bovine milk under supervision of Pharming B.V., Leiden, The Netherlands, purified by CLB, Amsterdam, and filled and finished by Chiron, Amsterdam, The Netherlands. The sterile drug solution was packed in glass vials. All medication was stored at -20°C. Heparin was manufactured by Leo Pharmaceutical Products B.V. and was stored at the pharmacy department of Pharma Bio-Research Group B.V.

Human lactoferrin was purified from the milk of transgenic bovine using the following method. Raw milk containing 0.5 – 5.0 g human lactoferrin /l was pooled and processed in batches of 200 l. Raw milk was defatted by centrifugation and skimmed milk was stored at < -20°C. A thawed 200 l batch of hLF containing skimmed milk was incubated with 10% (w/v) S Sepharose (Pharmacia) for 12 ± 4 hours at 4°C. Beads were allowed to settle for at least 5 hours and the supernatant was removed. The Sepharose was then washed batchwise with 6 x 1 volume 20 mM Na-phosphate pH 7.5, 0.1 M NaCl, and 7 x with 1 volume 20 mM Na-phosphate pH 7.5, 0.4 M NaCl. A BPG 300 column

(Pharmacia) was packed with the S Sepharose containing the bound hLF and washed with 3 volumes of 20 mM Na-phosphate pH 7.5, 0.4 M NaCl, at 42 l/h. The column was eluted with 20 mM Na-phosphate pH 7.5, 1.0 M NaCl, at 42 l/h. The S Sepharose eluate was concentrated to 10 l and buffer exchanged to 20 mM Na-phosphate pH 7.5, 0.1 M NaCl using Hemoflow (Fresenius, 30kD cut-off). The preparation was filtrated (0.22 μm Millipore) and subjected to virus inactivation by adjusting to 1% (w/v) Tween 80 and 0.3% (w/v) tri-n-butyl phosphate and incubating for 15 ± 3 hours at 25 ± 1°C. The virus inactivated sample was loaded at 42 l/h onto a Q Sepharose column (Pharmacia), 180 x 80, serially connected to a Macroprep High S Support (MPHS, Biorad) 300 x 15. Both columns were equilibrated in 20 mM Na-phosphate pH 7.5, 0.1 M NaCl. The columns were washed with 10 litres of 20 mM Na-phosphate pH 7.5, 0.1 M NaCl at 42 l/h. The Q Sepharose column was disconnected and the MPHS column was washed with 40 volumes of 20 mM Na-phosphate pH 7.5, at 126 l/h. The MPHS column was stepwise eluted with first 8 volumes of of 20 mM Na-phosphate pH 7.5, 0.4 M NaCl, at 42 l/h, and then 3 volumes of of 20 mM Na-phosphate pH 7.5, 0.5 M NaCl, at 42 l/h. Fractions were tested for human lactoferrin content and for bovine lactoferrin impurity. Human lactoferrin containing fractions free of bovine lactoferrin were pooled and concentrated to 10 litres with Hemoflow (Fresenius, 30 kD cut-off). The concentrate was filtrated over 2 serially placed Planova 15 N filters (Asahi, 15 nm, 1 m^2) for virus removal using a pressure of 0.5 bar. Again using the Hemoflow (Fresenius, 30 kD cut-off) the hLF preparation was concentrated to 9% and buffer exchanged to saline (0.9% NaCl). Finally the hLF preparation was sterile filtrated (Millipore, 0.1 μm) and stored at < -65°C.

Overall Study Design and Plan

The trial was a double blind, randomized, parallel group, controlled, ascending single intravenous dose, single center study and was conducted in 31 healthy male volunteers (Group I: n=4; Group II-IV: n=9). Subjects were randomized into group I (rhLF) or into one of three groups on a 4:4:1 basis (4 heparin + rhLF, 4 heparin placebo + rhLF, heparin + rhLF placebo).

Treatments Administered

In Group I four subjects received 2.5 mg/kg rhLF. In Groups II-IV subjects were randomized to either the previous rhLF dose following heparin infusion, the next rhLF

dose following placebo heparin or to heparin followed by placebo rhLF. For an overview of the groups, subject numbers and medication see Table 9.4.1-1.

Table 9.4.1-1 Treatment schedule

Group	Subjects	Treatment
I	01-04	2.5 mg/kg rhLF
IIa	08, 10, 12, 13	Heparin + 2.5 mg/kg rhLF
IIb	05, 06, 07, 11	Placebo heparin + 10 mg/kg rhLF
IIc	09	Heparin + placebo rhLF
IIIa	14, 16, 17, 20	Heparin + 10 mg/kg rhLF
IIIb	15, 18, 19, 22	Placebo heparin + 30 mg/kg rhLF
IIIc	21	Heparin + placebo rhLF
IVa	23, 25, 28, 29	Heparin + 30 mg/kg rhLF
IVb	26, 27, 30, 31	Placebo heparin + 60 mg/kg rhLF
IVc	24	Heparin + placebo rhLF

Subjects of Group I received on day 1 at t=0 a single dose of 2.5 mg/kg rhLF over a period of 10 minutes. Subjects of Groups II-IV received on day 1 at t=-15 minutes a single dose of heparin (15000 Units) or heparin placebo intravenously over a period of 5 minutes. At t=0 subjects received a single dose of rhLF or placebo-rhLF administered intravenously over a period of 10 minutes.. After a preceding dose was demonstrated to be safe, the next group received the following single dose.

Pharmacodynamic and pharmacokinetic assessments

To examine the efficacy of rhLF to neutralize the anticoagulation effect of heparin, the, pharmacodynamic parameters, including APTT, platelet aggregation, transferrin and total iron in blood samples, were reported. These parameters were used for evaluation of efficacy of rhLF administration to neutralizing the anticoagulant action of heparin.

Pharmacokinetic parameters determined from plasma concentration-time data for rhLF were:

C_{max} maximum plasma concentration;

t_{max} time attain first maximum plasma concentration;

$t_{½}$ terminal elimination half-life;

AUC_{0-t} area under the plasma concentration-time curve up to time t_{last}, where t_{last} is the last time point with a concentration above the lower limit of quantitation (log-linear trapezoidal rule);

$AUC_{0-\infty}$ total AUC after extrapolation from time x to time infinity, $(AUC_{0-t} + c/\lambda_z)$, where c is the approximated concentration at t_{last} using the regression results of λ_z.

Analysis of the plasma samples was performed using a validated enzyme-linked immunosorbent assay (ELISA) method in the concentration and a Western Blot method.

For APTT a 3 ml blood sample was collected in a Na-citrate tube. Blood was kept on ice until centrifugation at 3000 rpm for 10 minutes at 4°C. For platelet aggregation a 10 ml blood sample was collected in a Na-citrate tube. Blood samples were taken from a non-compressed arm, blood was not allowed to be kept on ice during handling. Samples were centrifuged at 18°C for 10 minutes at 1200 rpm. Following centrifugation, platelet-rich plasma was removed. The remaining of the sample was centrifuged at 4000 rpm for 10 minutes at 18°C, subsequently platelet-poor plasma was removed.

For assessment of transferrin and total iron, 2.5 ml blood was collected. Blood was centrifuged at 4°C for 10 minutes at 3000 rpm.

Blood samples were collected for the analysis of rhLF plasma concentrations. Blood samples (3 ml each) were collected at regular intervals on day1 at 0, 5, 10, 20, 30, 60, 90, 120, minutes, 4, 8, 24 and 48 h. Blood samples were collected into tubes containing EDTA (final concentration of 10 mM) and within 10 minutes centrifuged at 3000 rpm for 20 minutes at 4°C. Samples were stored at –70°C until analysis. The bioanalysis was performed by Pharma Bio-Research Group BV, Assen, The Netherlands.

Post-study screening

On day 7 and 22 subjects returned to the clinical research unit for post-study screening comprising: physical examination; vital signs, ECG; APTTassessment; platelet aggregation and bleeding time; safety biochemistry, hematology and urinalysis; blood sampling for anti body measurement against hLF and bLF; adverse events and concomitant medication recording.

Safety assessments

Adverse events, vital signs, ECG-recordings, physical examination, clinical laboratory parameters, FEV_1, Ivy-bleeding time, and antibody response against human lactoferrin (hLF) and bovine lactoferrin (bLF) were recorded as safety assessments.

III. Results

Heparin followed by placebo rhLF, 2.5 or 10 mg/kg rhLF resulted in prolonged APTT values above the ULQ (249 seconds) from t=0 until t=4 h post medication. After t=4 h, the median APTT values decreased and returned to baseline at t=8 h post medication. The heparin-induced increase in APTT is diminished by 30 mg/kg rhLF. Heparin followed by 30 mg/kg rhLF resulted in prolonged APTT values above ULQ at t = 0 h, decreased immediately to about 75 seconds after rhLF administration, and were back to baseline at t = 8 h post medication. Administration of rhLF after placebo-heparin treatment did not affect APTT values throughout the study period.

Heparin administration seemed not to have an effect on the platelet aggregation. Median day -1 to t=0 values showed a decrease of about 15% in the four groups without heparin administration and a decrease of about 3-10% in de heparin treated groups before rhLF administration. Considerable variation in median values was observed during 4h after administration of rhLF or placebo-rhLF. No trends could be observed. Administration of rhLF after placebo-heparin seemed not to have an effect on platelet aggregation. Descriptive statistics are presented in Section 14.2.1-4.

Heparin administration did not affect the concentrations of transferrin and total iron. However, groups treated with rhLF (2.5, 10 and 30 mg/kg) and heparin showed higher total iron values as compared to the group treated with heparin alone, although this elevation is in the range of individual differences. Administration of rhLF did not affect transferrin concentrations. Administration of rhLF after placebo-heparin did neither affect transferrin nor total iron throughout the study period. Descriptive statistics are presented in Section 14.2.1-5 and 14.2.1-6.

In summary, heparin-induced increments (>10 times baseline values) in blood coagulation time, as measured by APTT, in healthy volunteers could shortly and partially be returned to levels of 2.5 times baseline values after administration of rhLF, but only at the dose of 30 mg/kg rhLF. This effect occurred at 10 minutes post dose. All other doses of rhLF, i.e. 2.5 and 10 mg/kg did not show any effect upon APTT values. In all four

treatment groups with heparin, the APTT values returned to baseline values at 8 h post dose. Other dynamic parameters such as platelet aggregation, plasma transferrin and total iron concentration, after heparin treatment, were not significantly affected by rhLF administration.

Considering the total number of subjects participating in this study, very few adverse events were observed. Three moderate intensity adverse events occurred, of which two were classified as being possibly related to medication. These included an allergic reaction and nausea. Ivy bleeding time was not significantly affected by heparin and / or rhLF treatment.

IV. Discussion

The highest dose of recombinant human Lactoferrin (30 mg/kg) given in combination with heparin used in the present study was capable of partially inhibiting the effect of heparin on blood coagulation time within 10 minutes. Administration of 15000 U heparin i.v. alone induced a sharp increase in APTT from baseline values (approximately 30 seconds) to values above the upper limit of quantitation (249 seconds). This lasted for 120 minutes after placebo, 2.5 or 10 mg/kg rhLF administration. RhLF administration at a dose of 30 mg/kg was able to partially block the effect of heparin on blood coagulation, resulting in a sharp decrease in APTT immediately after rhLF administration to approximately 2.5 times baseline values (approximately 75 seconds). However, after the initial blocking effect of rhLF on the heparin-induced increase in APTT, the APTT values increased again to approximately 100 seconds. The return to baseline values was only achieved at t=8 h post medication. The increase in APTT at t=2 h, following the initial sharp decrease after 30 mg/kg rhLF administration might be the result of clearance of rhLF from the circulation by metabolic mechanisms, whereas the subsequent decrease in APTT at t=4–8 h is probably caused by the clearance of heparin from the circulation.

The present study shows maximal heparin activity until t=2 h post dose (i.e. 135 minutes post heparin administration). Animal studies revealed total clearance of 25 mg/kg rhLF from the blood within 3 hours, and a half-life time of 45 minutes (a comparison with pharmacokinetic data derived from the present study is required, but these data are not available at this moment).

A complete reversal of the anti-coagulating action of heparin by rhLF can be achieved by administration of a higher single dose of rhLF or by administration of rhLF

as a maintenance dose. If rhLF forms irreversible complexes with heparin, it is preferable to increase the dose of a single rhLF bolus infusion. However, if rhLF forms reversible complexes with heparin, a maintenance dose of rhLF infusion is preferred.

Other dynamic parameters such as platelet aggregation and plasma iron concentration measured after heparin treatment, were not significantly affected by rhLF administration.

Only a limited number of subjects experienced any adverse events and even these were minor. First, in Group IVb (placebo-heparin in combination with 60 mg/kg rhLF), two subjects experienced adverse events. One subject suffered a short but moderate period of nausea, duration 62 minutes. This reaction was thought to be possibly related to medication. The same subject also experienced a temperature changed sensation, possibly related and a pharyngitis, not related to medication. Another subject complained of headache and vision abnormality, both possibly related to medication and a myalgia not related to medication. Second, in Group IIIb (placebo-heparin in combination with 30 mg/kg rhLF) one subject experienced an allergic reaction. This event included urticaria, difficulty in breathing, conjunctivitis and Quinke's edema. This event was classified as being possibly related to medication. Other mild adverse events occurred throughout the duration of the study, but were mainly unrelated to medication

It can be concluded that the intravenous administration of rhLF alone and in combination with heparin is safe.

It will be apparent from the foregoing that the invention includes a variety of uses. These usages include the use of high dosages of lactoferrin for the manufacture of a medicament for parenteral administration to a patient for the treatment or prophylaxis of a disease, such as an infectious disease or inflammation. For the purposes of clarity and understanding, the invention has been described in these examples and the above disclosure in some detail. It will be apparent, however, that certain changes and modifications can be practiced within the scope of the appended claims. All publications and patent filings = cited in the present application are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted.

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CLAIMS

1. Use of lactoferrin, or a fragment or variant thereof, in the preparation of a medicament for the treatment or prophylaxis of a disorder selected from the group consisting of an infectious disease, an inflammatory disease, and an excess of heparin.

2. A use according to any one of claims 1 or 2, wherein the medicament is suitable for parenteral adminstering a dosage of at least 130 nmol lactoferrin, or a fragment or variant thereof, per kg body weight.

3. A use according to any one of the preceeding claims, wherein the administering is performed by intravenous injection.

4. A use according to any one of the preceeding claims, wherein the dosage is 260-1300 nmol/kg.

5. A use according to any one of the preceeding claims, wherein lactoferrin is intact human lactoferrin.

6. A use according to any one of claims 1 - 4, wherein the lactoferrin fragment is a fragment comprising amino acids 1-47 of SEQ ID No. 1.

7. A use according to claim 6, wherein the lactoferrin fragment is a fragment comprising at least 6 but no more than 27 contiguous amino acids from the N-terminal segment of human lactoferrin in SEQ ID NO:1, wherein the N-terminus of said polypeptide is residue 1 of SEQ ID NO:1.

8. A use according to any one of claims 1 - 4, wherein the lactoferrin fragment is a fragment comprising at least 7 contiguous amino acids of SEQ ID NO:1.

9. A use according to any one of claims 1 - 4, wherein the lactoferrin variant a variant that binds heparin with lower affinity than does natural lactoferrin.

10. A use according to claim 9, wherein the lactoferrin variant is hLF-2N, hLF-3N, hLF-4N, or hLF-5N.

11. A use according to any one of claims 1 - 4, wherein the lactoferrin variant is between about 3% and about 100% saturated with iron.

12. A use according to claim 11, wherein the lactoferrin variant is at least about 95% saturated with iron.

13. A method of treating a patient, comprising parenterally administering a dosage of lactoferrin or a fragment or variant thereof, of at least 130 nmol/kg body weight to the patient.

14. The method of claim 13, wherein the administering is performed by intravenous injection.

15. A method according to claims 13 or 14, further comprising administering a second dosage of lactoferrin or a fragment or variant thereof, orally to the patient.

16. A method according to any one of claims 13 - 15, wherein the lactoferrin is intact human lactoferrin and the dosage is at least 30 mg/kg.

17. A method according to any one of claims 13 - 16, wherein the dosage is 260-1300 nmol/kg.

18. A method according to any one of claims 13 - 17, wherein the patient suffers or is susceptible to a disorder selected from the group consisting of an infectious disease, an inflammatory disease, and an excess of heparin and the dosage is sufficient to prevent, or treat the disorder.

19. A method according to any one of claims 13 - 18, wherein the patient is substantially free of side-effects response to administration of the lactoferrin.

20. A method according to any one of claims 13 - 19, wherein the dosage is administered daily for a period of at least a week.

21. A method according to any one of claims 13 - 20, wherein the lactoferrin is intact lactoferrin.

22. A method according to any one of claims 13 - 21, wherein the lactoferrin is intact human lactoferrin.

23. A method according to any one of claims 13 - 19, wherein the lactoferrin fragment is a fragment comprising amino acids 1-47 of SEQ ID No. 1.

24. A method according to claim 23, wherein the lactoferrin fragment is a fragment comprising at least 6 but no more than 27 contiguous amino acids from the N-terminal segment of human lactoferrin protein in SEQ ID NO:1, wherein the N-terminus of said polypeptide is residue 1 of SEQ ID NO:1.

25. A method according to any one of claims 13 - 19, wherein the lactoferrin fragment is a fragment comprising at least 7 contiguous amino acids of SEQ ID No:1.

26. A method according to any one of claims 13 - 19, wherein the lactoferrin fragment is a fragment comprising at least 6 but no more than 24 contiguous amino acids.

27. A method according to any one of claims 13 - 19, wherein the lactoferrin variant is a variant that binds heparin with lower affinity than does natural lactoferrin.

28. A method according to claim 27, wherein the lactoferrin variant is hLF-2N, hLF-3N, hLF-4N, or hLF-5N.

29. A method according to any one of claims 13 - 19, wherein the lactoferrin variant is between about 3% and about 100% saturated with iron.

30. A method according to claim 29, wherein the lactoferrin variant is at least about 95% saturated with iron.

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NUTRITION AND CANCER, 38(2), 192–199

Orally Administered Lactoferrin Exerts an Antimetastatic Effect and Enhances Production of IL-18 in the Intestinal Epithelium

Tetsuya Kuhara, Masaaki Iigo, Takehito Itoh, Yoshihiko Ushida, Kazunori Sekine, Nobuyuki Terada, Haruki Okamura, and Hiroyuki Tsuda

Abstract: *The effects of oral administration of bovine lactoferrin (bLF) and its hydrolysate on the lung colonization by colon 26 carcinoma were investigated. At doses of 100 or 300 mg/kg/day for seven successive days, bLFs demonstrated a significant inhibitory effect on experimental metastasis, which indicated effectiveness before and after tumor implantation. Oral administration of bLFs augmented $CD4^+$, $CD8^+$, and asialoGM1$^+$ cells in the spleen and peripheral blood. Their cytotoxic activities against Yac-1 and colon 26 carcinoma were enhanced by bLF. In the small intestinal epithelium, $CD4^+$ and $CD8^+$ cells were markedly increased, and, simultaneously, enhanced production of interleukin-18 (IL-18) was confirmed in the intestinal epithelial cells. In this model, intravenous injection of murine IL-18 showed significant inhibition of the lung colonization by colon 26 carcinoma. These results suggested that inhibition of experimental metastasis by oral administration of bLF and pepsin hydrolysate of bLF might be due to enhanced cellular immunity, presumably mediated by enhanced IL-18 production in the intestinal epithelium.*

Introduction

Lactoferrin (LF) is an iron-binding glycoprotein of ~80 kDa, present in milk, epithelial secretions, and the second granules of neutrophils. The amino acid sequence of LF is highly conserved among mammalian species (1). This molecule has a number of physiological functions, including antimicrobial activity (2,3), stimulatory and inhibitory effects on cell growth (4), and extensive immune system-modulating activities, e.g., $CD4^+$ T cell maturation in thymocytes (5), inhibition of Th1, but not Th2, response (6), augmentation of natural killer (NK) and lymphokine-activated killer (LAK) cell cytotoxicity (7), enhancement of polymorphonuclear leukocyte and phagocytic cell activity (8), and regulation of cytokine production (9,10). Although some of the proposed biological functions of LF hinge on its iron-binding capacity, other functions include binding to the lipid-A part of lipopolysaccharide (11), ribonuclease enzymatic activity (12), and DNA binding and transcriptional activation (10,13). LF receptors have been suggested to be present in some tissues and cells, but these molecules have not been identified (14).

Although LF has been identified as a mucosal host defense mediator and inflammatory immune modulator, it has also been reported that administration of human (hLF) or bovine LF (bLF) inhibited the growth of solid tumors and metastases in mice (15,16). In these cases, LFs were administered intraperitoneally, intravenously, or subcutaneously, and enhanced NK activity by hLF and antiangiogenesis activity of bLF have been suggested to explain its antitumor functions. It is important to determine whether oral administration of LF is associated with its reported antitumor and antimetastatic activities, because LF obtained in the diet is too large to be absorbed *via* the intestine. Recently, we found that bLF prevents colon carcinoma induced by azoxymethane in rats when given as a dietary supplement (17). This experiment also showed that oral ingestion of bLF enhanced NK activity, although obvious absorption of bLF into the circulation was not detected. In the present study, we examined the effects of orally administered bLF on antimetastatic host defense through the intestinal immune system, especially interleukin (IL)-18 production. IL-18 is a potent stimulator of interferon-γ (IFN-γ) production by T cells and also augmented NK cell (18) and CTL (19) activity. It has been suggested that intestinal epithelial cells may be the main producers of IL-18 (20). We report here that oral administration of bLF induced IL-18 production in the small intestine, which may modulate immunologic function, and inhibited formation of lung metastatic colonies.

Materials and Methods

Animals and Tumors

Specific pathogen-free male inbred Balb/c and athymic nude (Balb/c *nu/nu*) mice were obtained from Charles River

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(Kanagawa, Japan). The mice were six weeks of age at the time of the experiments. All studies were conducted in accordance with the guidelines for the care and use of laboratory animals of the National Cancer Research Institute, Japan.

The Co26Lu cell line was isolated from high lung-metastatic colon carcinoma 26 and maintained in RPMI 1640 medium supplemented with 100 μg/ml of streptomycin, 100 U/ml of penicillin, and 10% fetal bovine serum under an atmosphere of 5% CO_2 at 37°C.

Test Compounds

bLF was prepared from the whey protein fraction of cow's milk by the procedure reported previously (21). Pepsin hydrolysate of bLF (bLFH) was prepared as described previously (17).

Antimetastatic Experiments

Aliquots of Co26Lu cells [5×10^4/0.2 ml in phosphate-buffered saline (PBS)] were injected into the tail vein of Balb/c and/or athymic nude mice. Balb/c mice injected intraperitoneally with anti-asialoGM1 antibody (Wako Pure Chemical, Osaka, Japan) at 25 μg/animal one day before tumor inoculation were also examined. bLF and bLFH (30, 100, and 300 mg/kg) were administered orally in physiological saline (0.2 ml/20 g body wt) *via* a stomach tube for seven consecutive days from Day 7 before or Day 1 after tumor cell injection. After the end of the experiments (Day 11), the mice were sacrificed, and lungs were removed and fixed in acetone to determine numbers of macroscopic lung colonies. These experiments were repeated twice with groups of five to six mice. In another series, recombinant murine IL-18 was injected intravenously one day before or after tumor inoculation at doses of 10, 25, and 50 μg/kg.

Immunofluorescence Analysis

Peripheral white blood cells (WBCs) and spleen cells were collected from control and/or tumor-bearing mice 24 hours after seven administrations of bLF and bLFH. Intestinal intraepithelial cells were isolated by the method of Ishikawa and co-workers (22). The cells were immersed in 10 mM tris(hydroxymethyl)aminomethane-buffered solution containing 0.14 M NH_4Cl to lyse red blood cells and then washed twice in PBS and suspended in RPMI 1640 medium. The following antibodies were used for immunofluorescence staining: anti-mouse CD16/32 (clone 2.4G2, Fc Block), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3e (145-2C11), FITC- or phycoerythrin (PE)-conjugated anti-CD4 (RM4-5), FITC- or PE-conjugated anti-CD8a (53-6.7) (Pharmingen, San Diego, CA). FITC-conjugated anti-rabbit immunoglobulin (Ig) (G + L, Tago, Burlingame, CA) was used as a second antibody against anti-asialoGM1 rabbit serum. Relative fluorescence intensities of single- or two-color staining were then measured with a FACScan (Becton-Dickinson, San Jose, CA), and the data were analyzed using a Consort 30 (Becton-Dickinson).

Measurement of NK Cell Activity and Cytotoxic Activity in WBCs and Spleen Cells

The NK cell activity of WBCs and spleen cells was measured using ^{51}Cr sodium chromate-labeled Yac-1 cells. Effector cells obtained on Day 8 in the experimental metastasis model (intravenously Co26Lu-inoculated mice) were diluted serially and incubated in 96-well round-bottomed microtiter plates with 5×10^3 Yac-1 cells for four hours at 37°C. Percent cytotoxicity was calculated as follows: 100 × (count of experimental release − count of spontaneous release)/(count of total release − count of spontaneous release). Cytotoxic activity of spleen cells against Co26Lu was measured using a LIVE/DEAD Eukolight CMC kit (Molecular Probes, Eugene, OR). Percent cytotoxicity was calculated as follows: 100 × (dead cells/200 visible cells).

Determination of Serum bLF

Blood samples were collected from the portal vein and heart at one, two, three, and four hours after final administration of bLF. Enzyme-linked immunosorbent assay with a specific monoclonal antibody developed in our laboratory was used to determine bLF concentrations in serum. The minimal detectable dose was ~44 pg/ml.

Measurement of IL-18 Expression in the Small Intestine

For detection of IL-18 mRNA in the intestinal epithelium, bLF was administered orally to tumor-bearing mice on Days 1–7, and the mice were killed on Day 8. The small intestine was excised, and intestinal epithelial cells were harvested. These samples were investigated by Northern blot analysis. Aliquots of 20 μg total RNA isolated using RNAzol-B (Teltest, Friendswood, TX) were electrophoresed, transferred onto nylon membranes, and hybridized with a ^{32}P-labeled mouse IL-18 cDNA probe using a Megaprime DNA labeling kit and Rapid-hyb buffer (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The same membranes were stripped and rehybridized with a glyceraldehyde 3-phosphate dehydrogenase probe. The IL-18 probe was designed according to the sequence reported by Okamura and colleagues (18). IL-18 production in the intestine was detected by immunoblotting analysis. The intestinal epithelium was homogenized in lysis buffer consisting of PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate containing 10 μg/ml of phenylmethylsulfonyl fluoride. Homogenates were clarified by centrifugation at 15,000 rpm for 10 minutes, and the supernatants were collected and stored at −80°C until testing. Protein concentrations of the extracts were measured using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with rabbit anti-mouse IL-18 polyclonal IgG (23). For immunohistochemical analysis, whole intestines were fixed in Zamboni's fixative and embedded in paraffin. After deparaffinization, tissue sections were treated with 3% H_2O_2

in 50% methanol and incubated with normal horse serum. IL-18-producing cells were immunostained using a rat anti-mouse IL-18 monoclonal antibody. Immunoreactivity was detected using biotinylated rat IgG and a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

Statistical Analysis

Numbers of lung colonies were analyzed by the Mann-Whitney *U* test. Other data were evaluated using Dunnett's modification of Student's *t*-test.

Results

Antimetastatic Effects of bLF and bLFH Administered Orally

Table 1 summarizes the effects of bLF and bLFH on Co26Lu lung colonization. Lung colonization was inhibited by oral administration of bLF for seven days after intravenous implantation of tumor cells (Table 1A). A significant decrease ($p < 0.01$) in colonies was observed at a dose of 300 mg/kg. bLFH also significantly inhibited lung colonization ($p < 0.01$) at doses of 100 and 300 mg/kg. When bLF or bLFH was administered for seven days before implantation of tumor cells, lung colony formation was significantly decreased ($p < 0.001$) at 300 mg/kg (Table 1B), similar to the effects of administration after implantation. Next, we examined whether these compounds exerted their antimetastatic effects directly or indirectly. As shown in Table 1C, whereas the number of lung colonies was low because of the relatively high NK activities in athymic nude mice, bLF and bLFH did not significantly decrease lung colonization. Furthermore, bLF did not reduce lung colonization in anti-asialoGM1 antibody-treated mice. However, bLFH induced significant decreases ($p < 0.01$) in the number of Co26Lu lung colonies (Table 1D).

Table 1. Effects of Oral Administration of bLF and bLFH on Number of Experimental Co26Lu Lung Metastatic Lesions[a]

	Number	*P* Value
A: Balb/c mice		
Nontreated	72.0 (59–110)	
Treated		
LF		
30 mg/kg/day	60.0 (26–74)	0.062
100 mg/kg/day	59.0 (43–93)	0.159
300 mg/kg/day	47.0 (30–60)	0.006
LFH		
30 mg/kg/day	62.0 (46–251)	0.406
100 mg/kg/day	48.0 (8–66)	0.007
300 mg/kg/day	37.0 (9–44)	0.002
B: before vs. after tumor inoculation		
Nontreated	146.0 (80–232)	
Treated		
LF		
Pre	70.5 (55–113)	<0.001
Post	37.0 (22–97)	<0.001
LFH		
Pre	66.5 (32–122)	<0.001
Post	40.5 (20–79)	<0.001
C: Balb/c-nu/nu mice		
Nontreated	31.0 (20–92)	
Treated		
LF (300 mg/kg/day)	40.0 (13–75)	0.896
LFH (300 mg/kg/day)	22.0 (13–61)	0.067
D: Balb/c mice treated with anti-asialoGM1		
Nontreated	119.0 (106–181)	
Treated		
LF (300 mg/kg/day)	142.0 (35–167)	0.654
LFH (300 mg/kg/day)	77.0 (29–109)	0.004

a: 12 Balb/c mice (A, B, and D) and 10 Balb/c athymic nude (*nu/nu*) mice (B) were inoculated intravenously with 5×10^4 Co26Lu cells. Test compounds were administered by mouth from 1 day after (Post) tumor inoculation (A, C, and D) and/or from 7 days before (Pre) tumor inoculation (B) for 7 days. To eliminate NK activity, anti-asialoGM1 antibody (25 μg/animal) was injected intraperitoneally 1 day before tumor inoculation (D). On Day 11, animals were sacrificed, and lungs were placed in acetone. Values were compared with those of nontreated controls by Mann-Whitney *U* test. Values in parentheses are ranges.

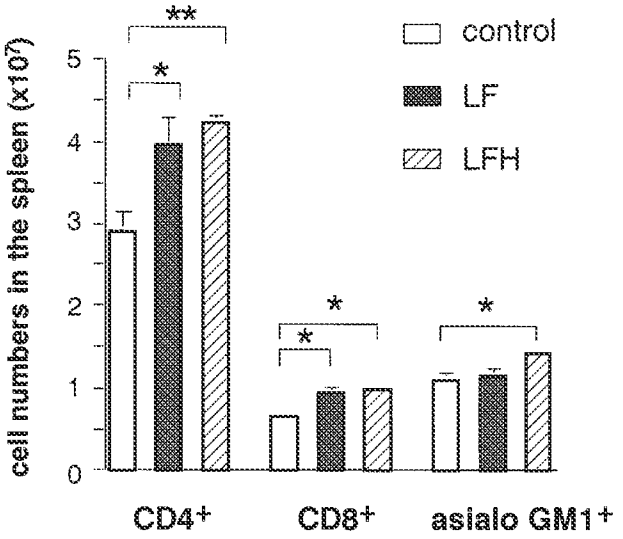

Figure 1. Effects of bovine lactoferrin (bLF)and pepsin hydrolysate of bLF (bLFH) on numbers of $CD4^+$, $CD8^+$, and asialoGM1$^+$ cells in spleen in non-tumor-bearing mice. bLF or bLFH was administered to 3 non-tumor-bearing animals per group at 300 mg/kg/day po for 7 successive days. At 24 h after last administration, spleen cells were prepared for immunofluorescence analysis. These experiments were repeated twice (total animal number was 6). *, $p < 0.05$; **, $p < 0.01$ compared with control by Dunnett's modification of Student's *t*-test.

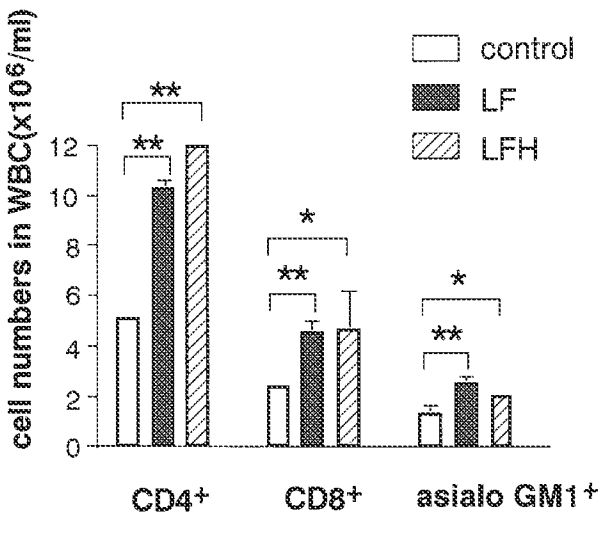

Figure 2. Effects of bLF and bLFH on numbers of $CD4^+$, $CD8^+$, and asialoGM1$^+$ cells in peripheral blood of tumor-bearing mice. bLF or bLFH was administered to 3 tumor-bearing animals per group as described in Figure 1 legend. At 24 h after last administration, animals were placed under light ether anesthesia, blood samples were collected from heart, and white blood cells (WBCs) were prepared for immunofluorescence analysis. These experiments were repeated twice (total animal number was 6). *, $p < 0.05$; **, $p < 0.01$ compared with control by Dunnett's modification of Student's *t*-test.

Influence of Oral Administration of bLF and bLFH on Peripheral $CD4^+$, $CD8^+$, and AsialoGM1$^+$ Cell Populations

bLF and bLFH administration indicated the inhibitory effects against lung colony formation before and after tumor implantation, and these effects disappeared to some extent in immune-deficient mice. Next, we investigated whether oral administration of bLF and bLFH had immune modulating functions in non-tumor-bearing or tumor-bearing mice. In non-tumor-bearing mice, oral administration of bLF and bLFH significantly increased the numbers of $CD4^+$, $CD8^+$, and asialoGM1$^+$ cells in the spleen (Figure 1). However, in tumor-bearing mice, the spleen cell numbers decreased, so each population showed little change. On the other hand, these populations in the peripheral blood in tumor-bearing mice were significantly augmented by bLF and bLFH (Figure 2), although they did not influence the percentages of blood monocytes or neutrophils calculated by examination of Wright-Giemsa-stained smears (data not shown).

For determination of NK activity, we used freshly isolated WBCs and spleen cells from tumor-bearing mice as effectors and Yac-1 cells as target cells (Figure 3A). bLF significantly augmented NK activity of both effectors at an effector-to-target ratio of 30:1. Furthermore, Co26Lu cells, which is an inoculum used as an *in vivo* experimental metastasis model, were used for measurement of cytotoxicity of spleen cells (Figure 3B). bLF administration to tumor-bearing mice significantly enhanced cytotoxicity against Co26Lu cells at effector-to-target ratios of 10:1 and 30:1.

Influence of Oral Administration of bLF on Intestinal Immunity

After the final oral administration of bLF, serum bLF concentrations in samples from the portal vein and heart were below the limits of detection by enzyme-linked immunosorbent assay (sampled after 1–4 h in 10 animals at each time point). These results suggested that bLF and bLFH may affect the immune system in the gastrointestinal tract. Figure 4 shows the increases in $CD4^+$ and $CD8^+$ populations in the intestinal intraepithelial lymphocytes isolated from bLF-treated mice, as determined by two-color staining using anti-CD3 (FITC) and anti-CD4 or CD8 (PE). Next, we investigated the effects of bLF and bLFH administration on the expression of IL-18 in the intestinal epithelium, because IL-18 is constitutively expressed at high levels in the intestinal epithelium (20), where it activates $CD4^+$, $CD8^+$ T cells, and NK cells (24) and stimulates IFN-γ production (18). As shown in Figure 5A, Northern blot analysis indicated that oral administration of bLF for three days markedly enhanced IL-18 mRNA expression in the small intestine, which showed a higher level of IL-18 mRNA expression than seven days of administration (data not shown). Figure 5B shows the results of immunoblotting analysis. Murine IL-18 is synthesized as proIL-18 (~24 kDa), which is cleaved by IL-1β-converting enzyme into the active form (~18 kDa) (23). In the samples of the intestinal epithelium, it was present mainly as proIL-18. Figure 5B indicates that bLF and bLFH enhanced the production of IL-18, and a three-day treatment regimen with both compounds was more effective than a seven-day treatment regimen. Figure 5C shows the results of immunohistochemical analysis of the small

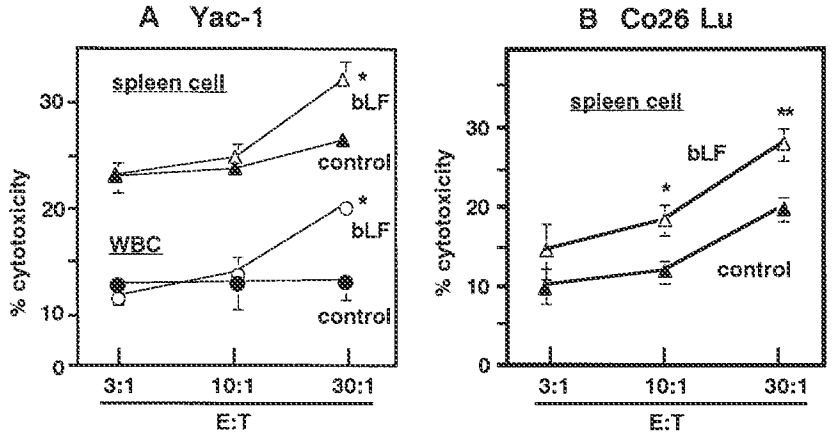

Figure 3. Effects of bLF on cytotoxicity of spleen cells and/or WBCs against Yac-1 and Co26Lu cells. A: spleen cells and WBCs were collected from tumor-bearing mice treated as described in Figure 2 legend and cocultured with 5×10^3 ^{51}Cr-labeled Yac-1 target cells for 4 h. ^{51}Cr release into supernatants of cultures at indicated effector-to-target ratios (E/T) was measured. B: results from fluorescent dye study. 3,3′-dioctadecyloxacarbocyanine perchlorate-labeled Co26Lu target cells were cocultured for 24 h. At 15 min after addition of propidium iodide, viability of target cells was measured by fluorescence microscopy. *, $p < 0.05$; **, $p < 0.01$ compared with control by Dunnett's modification of Student's *t*-test.

intestine, around the jejunum. The numbers of positively stained intestinal epithelial cells were increased by bLF compared with untreated controls.

Antimetastatic Effects of Murine IL-18

In our experimental lung metastasis model, we confirmed the direct effect of murine IL-18. Our results indicated that intravenous administration of 10, 25, or 50 μg/kg of recombinant murine IL-18 one day before or one day after inoculation significantly ($p < 0.01$) and dose dependently reduced lung colonies (25 μg/kg in both cases showed reduction by ~70%).

Discussion

In our previous study, we showed that oral administration of bLF and related compounds [bLFH and bovine lactoferricin (bLFcin): NH_2-terminal region of bLF consisted of 25 amino acids] markedly inhibited spontaneous lung metastasis formation by colon 26 carcinoma cells (25). In the present study, we examined the augmentation of antimetastatic host defense by oral administration of bLF and bLFH. Our results suggested that bLF and bLFH reduce lung metastasis by administration before as well as after tumor implantation and exerted cell-mediated cytotoxic effects through the induction of IL-18 production in the intestine.

Bezault and co-workers (15) reported that intraperitoneal administration of hLF suppressed the growth of implanted NIH/3T3 rpIA fibroblastoma and inhibited lung colonization by B16-F10 melanoma in an experimental metastasis model, and they suggested that these effects may be mediated by augmentation of NK activity. Moreover, Yoo and colleagues (16) reported that intraperitoneal or subcutaneous

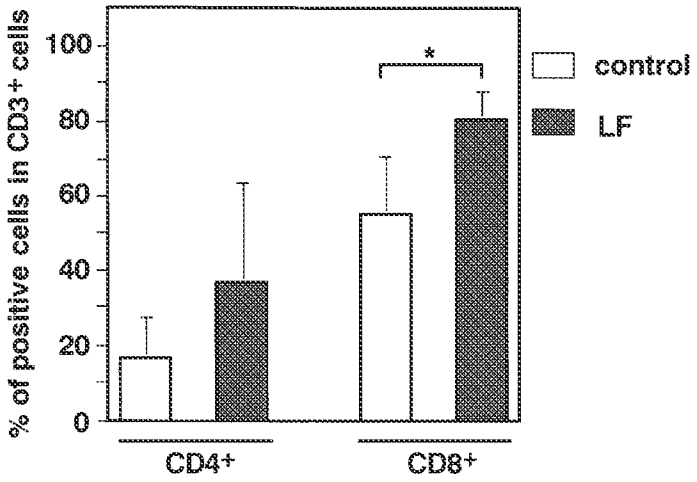

Figure 4. Effects of bLF on numbers of $CD4^+$ and $CD8^+$ cells in intestinal intraepithelial T lymphocytes. Animals (4/group) were treated as described in Figure 2 legend. Intestinal intraepithelial cells were collected by shaking and prepared for immunofluorescence analysis. *, $p < 0.05$ compared with controls by Dunnett's modification of Student's *t*-test.

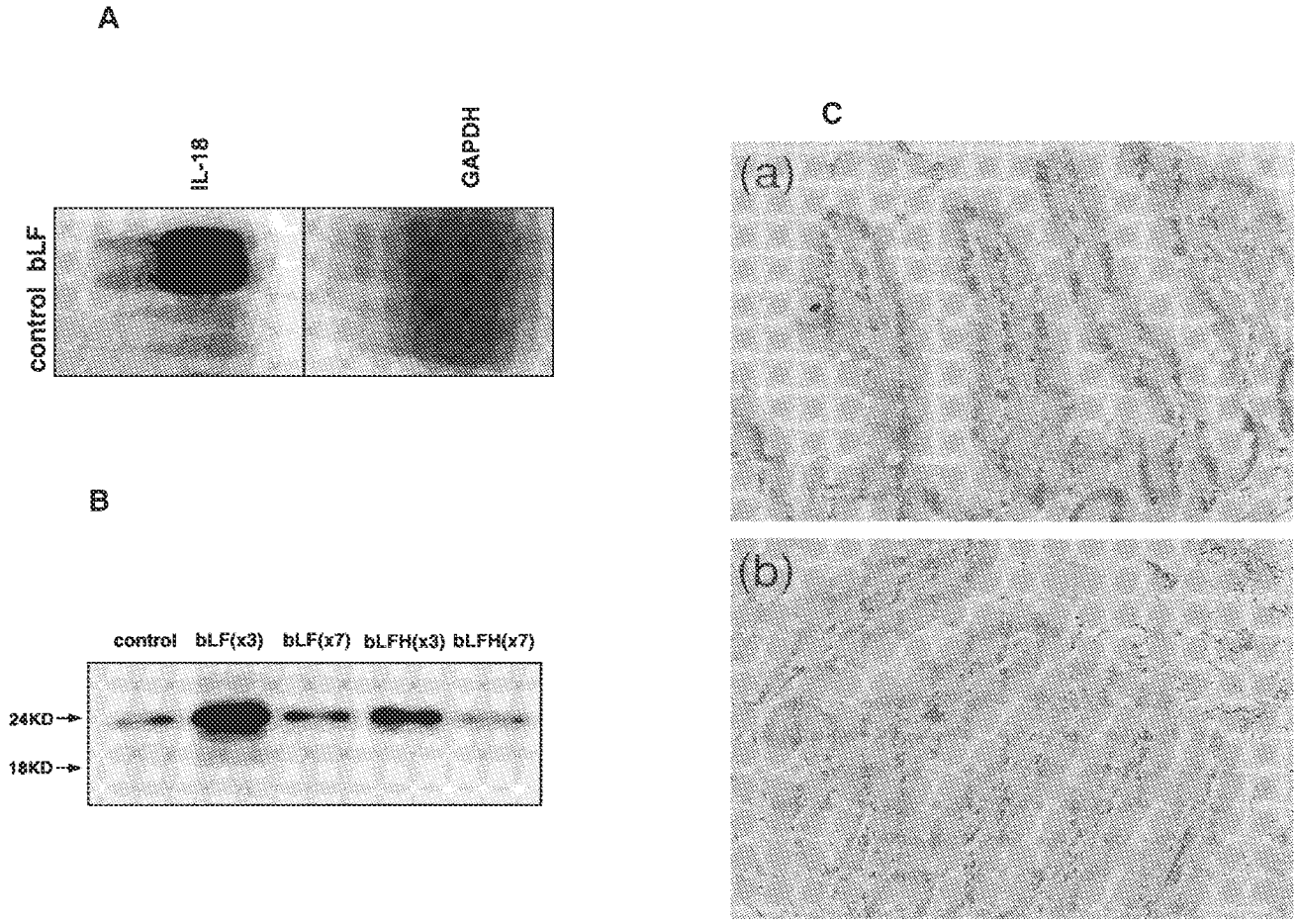

Figure 5. Effects of bLF and bLFH on interleukin-18 (IL-18) production in intestinal epithelial tissue. A: Northern blot analysis of murine IL-18 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression in intestinal epithelium collected from bLF-treated (po, 3 days) and control mice. B: immunoblot analysis of murine IL-18 in intestinal epithelium collected from bLF- and bLFH-treated (po, 3 and 7 days) and control mice. Aliquots of 100 μg of protein were applied to each lane. C: immunohistochemical analysis of murine IL-18 in intestinal epithelial cells collected from control (a) and bLF-treated mice (b). Magnification ×100.

administration of bLF and bLFcin inhibited lung metastasis of B16-BL6 melanoma and L5178Y-ML25 lymphoma cells in experimental and spontaneous metastasis models. They suggested that these effects might have been due to suppression of tumor-induced angiogenesis. In an *in vitro* experiment, LF showed immune-modulating effects including augmentation of NK and T cell-mediated cytotoxicity (7). However, LF cannot be absorbed as the undigested form, because it is a large molecule, so it is unlikely that it had direct effects in the present study. In addition, bLF and bLFH, even at 100 μg/ml, did not directly affect tumor cells in an *in vitro* experiment (data not shown). LF receptors have been suggested to exist in the intestinal brush borders (14). LF shows species-related differences in function, which may be dependent on receptor-binding capacities (14). Ingested LF might be taken up into intestinal epithelial cells and partly digested, and LF or related compounds may bind to DNA, leading to transcriptional activation (13) or downmodulation (10).

In the present study, a slight difference was observed between bLF and bLFH in the experiment using anti-asialoGM1 antibody-treated mice, in which the latter but not the former significantly reduced lung colony formation (Table 1D). bLFH consists of ~10-kDa peptides and includes bare bLFcin. As in the previous study, bLFcin inhibited lung colonization by Co26Lu cells, but in an experimental lung metastasis model it did not affect any immune response measured (data not shown). Inasmuch as bLFcin has been reported to show inhibitory effects on angiogenesis (16) and to induce apoptosis in THP-1 human leukemic cells (26), this 25-amino acid peptide itself appears to have functions different from those of bLF. Therefore, it has been suggested that bLFH has its own antimetastatic effects with additional effects of bLFcin and also that the region mainly responsible for stimulation of the antimetastatic immune response induced by bLF in the pepsin hydrolysate lies outside the NH_2-terminal 25 amino acids.

IL-18 markedly stimulates IFN-γ production in T cells, proliferation of stimulated T cells, and augmentation of NK activity in mice (18) and humans (27). IL-18 is produced by murine intestinal epithelial cells (20) and keratinocytes (28)

as well as macrophages/Kupffer cells (18). Thus IL-18 may be an important factor in local immune responses in the intestinal mucosa and skin. IL-18 was also shown to enhance antitumor activities through NK and $CD4^+$ T cell activation (29). We could not directly demonstrate a causal link between inhibition of lung colonization and enhanced production of IL-18 in the intestinal epithelium. On the one hand, we confirmed the direct effect of IL-18 in our experimental lung metastasis model. Therefore, it is obvious that this experimental lung metastasis model is sensitive to the effects of IL-18, and production of IL-18 may be important for bLF and bLFH to inhibit lung colony formation. The present study showed that bLF and bLFH administered orally induced the production of IL-18 in the intestinal epithelium more potently after three days of administration than after seven days, suggesting that timing and doses of administration of bLF are important.

LF may be thought of as a mucosal host defense mediator because of its presence in epithelial secretions as well as in milk. So, it is of interest whether LF and IL-18 exert functions in collaboration for the mucosal immunity and whether IL-18 production in local mucosal tissue, for example, the intestinal epithelium, influences not only mucosal immunity but the immune system of the whole body. The results of the present study indicated that oral administration of bLF and related compounds may enhance mucosal immunity, especially NK and T cell activity. Therefore, bLF might serve as an effective means for preventing metastasis.

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Title of Invention: Lactoferrin compositions and methods of wound treatment

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X. RELATED PROCEEDINGS APPENDIX

There are no related proceedings to this action, thus no copies of proceedings are provided.